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Cancer Drug Resistance

Review

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Auranofin and its analogs as prospective agents for the treatment of colorectal cancer

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Abstract

Today colorectal cancer (CRC) is one of the leading causes of cancer death worldwide. This disease is poorly chemo-sensitive toward the existing medical treatments so that new and more effective therapeutic agents are urgently needed and intensely sought. Platinum drugs, oxaliplatin in particular, were reported to produce some significant benefit in CRC treatment, triggering the general interest of medicinal chemists and oncologists for metal-based compounds as candidate anti-CRC drugs. Within this frame, gold compounds and, specifically, the established antiarthritic drug auranofin with its analogs, form a novel group of promising anticancer agents. Owing to its innovative mechanism of action and its favorable pharmacological profile, auranofin together with its derivatives are proposed here as novel experimental agents for CRC treatment, capable of overcoming resistance to platinum drugs. Some encouraging results in this direction have already been obtained. A few recent studies demonstrate that the action of auranofin may be further potentiated through the preparation of suitable pharmaceutical formulations capable of protecting the gold pharmacophore from unselective reactivity or through the design of highly synergic drug combinations. The perspectives of the research in this field are outlined.

Keywords: Auranofin, CRC, colorectal cancer, metallodrugs, anticancer agents, gold, gold-based drugs

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in Western countries and is a major worldwide health problem being one of the primary causes of cancer death[1]. Advances in the diagnosis and treatment of CRC brought a major effect in the management of this disease. Notably, in the last decades, screening programs and early diagnosis, together with new therapeutic options, allowed reducing CRC mortality considerably[2]. Despite this progress and the remarkable increase of knowledge about CRC biology and treatment, metastatic cases are still associated with a poor prognosis. In fact, the percentage of survival drops from ~65% to ~10% in the presence of metastatic CRC disease[3]. At least half of patients with colorectal cancer develop metastases[4], resulting in a poor outcome. The primary site for metastasis development is liver, representing the only site of metastasis in about one third of patients. Other common organs for distant metastases are lung, peritoneum, bone, brain, and spleen[4,5]. In CRC patients, the first therapeutic option is surgical resection, but the role of adjuvant chemotherapy (and chemoradiotherapy), in terms of disease-free survival and overall survival, is well recognized[6,7].

CRC is reputed as poorly chemo-sensitive, and for more than 30 years fluorouracil has been the reference drug. Fluorouracil can be administered both through continuous infusion line and through bolus administration. Recent reports evidenced however no significant differences in terms of prolonged survival rate[8,9]. Many efforts have been made to improve these results. Biochemical modulation of fluorouracil is one of the most interesting approaches to increase the therapeutic index of this compound. In metastatic disease confined to the liver, locoregional therapy through implantable pumps may be taken into account as well[10].

Until today, the standard therapy for CRC has relied on fluorouracil plus levamisole and/or calcium folinate (folinic acid)[6]. More recently, other treatment options have been introduced in the clinical practice. At present, there is increasing attention for the role of monoclonal antibodies (Mab) in CRC therapy. Among the various Mabs approved so far for CRC treatment, bevacizumab, panitumumab, and ramucirumab are the most commonly used[11]. Other clinical strategies rely on portal vein infusion of fluorouracil, alone or in combination with systemic therapy[12]. In rectal cancer, the best results are achieved by combining radiotherapy and chemotherapy. Recent reports support the use of induction or consolidation chemotherapy before surgery in locally advanced forms as well as total neoadjuvant treatment as the best therapeutic options[13]. In advanced colorectal cancer, a standard treatment has not been established yet, and different therapeutic options can be proposed according to the tumor staging and features. Typically, the first-line chemotherapy relies on the FOLFOX (leucovorin, 5-fluorouracil, and oxaliplatin) or CAPOX (capecitabine and oxaliplatin) protocol alone or in combination with Mab. At variance, the second-line approach also relies on compounds such as irinotecan and raltitrexed now entered in the clinical practice with encouraging results. For instance, FOLFIRI-based (leucovorin, 5-fluorouracil, and irinotecan) regimens are a common clinical practice, even in combination with antiangiogenic agents[14].

PLATINUM DRUGS IN CRC TREATMENT

Pt drugs play an important role in CRC treatment. Oxaliplatin is the only platinum anticancer drug in the clinical use for colorectal cancer, while cisplatin is not effective in the treatment of this tumor. The resistance to cisplatin in CRC is determined by several factors mostly related to the processing of cisplatin-induced DNA lesions by various biological actors. The tumor suppressor protein p53 is a key player in the cellular response to cisplatin. It is responsible for controlling the cascade of events leading to cell cycle

arrest/repair or apoptosis through transcription and activation of numerous p53-dependent DNA damage response genes. Accordingly, deactivation of p53 in CRC is involved in cisplatin chemoresistance. In this view, the DNA mismatch repair (MMR) process has a relevant role as well. Indeed, cisplatin-induced lesions are recognized by MMR. Conversely, oxaliplatin-induced lesions to DNA are not recognized. As a consequence, oxaliplatin shows cytotoxic and anticancer effects that are basically independent of the MMR process[15,16]. However, beyond the formation of adducts with DNA, which are extensively recognized as a key step for the pharmacological activity of platinum-based anticancer drugs[17,18], the different therapeutic indication between cisplatin and oxaliplatin was discussed by Bruno *et al.*[19] in a seminal paper published in 2017. In this article, the authors reported on the mechanisms behind the anticancer effects of platinum-based drugs. Although it is not clear to what extent the effect of DNA-independent pathways contributes to the anticancer profile compared to the toxic effect of DNA adducts formation, the above authors pointed at the ribosome biogenesis stress induced by oxaliplatin as a likely mechanism also contributing to the overall pharmacological activity. This finding is relevant because might be involved in the distinct clinical implementation of oxaliplatin compared with cisplatin[19].

As outlined above, Pt compounds, and oxaliplatin in particular, play a major role in the current medical treatment of CRC. Although being featured by a higher tolerability compared with cisplatin, oxaliplatin manifests a few relevant limitations such as relevant systemic toxicity and the frequent insurgence of resistance that may lead to eventual treatment failure. These limitations imply that the discovery of new drugs for colorectal cancer is absolutely mandatory and urgent. Here, we analyze specifically the chances of discovering new effective anticancer agents within the field of metal-based drugs.

METAL BASED DRUGS AS A SOURCE OF NOVEL ANTICANCER AGENTS CAPABLE OF OVERCOMING DRUG RESISTANCE: THE CASE OF GOLD COMPOUNDS

Platinum-based compounds are very effective only against a relatively limited number of tumor types and manifest at the same time some severe side effects (e.g., gastrointestinal toxicity, nervous system toxicity, and bone marrow suppression) that heavily limit their use[20-22]. In addition, intrinsic and acquired drug resistance may greatly reduce the efficacy of platinum drugs with a consequent poor prognosis[23]. For this reason, intense efforts are warranted to explore and identify novel anti-tumor metallodrugs that may replace platinum compounds for specific therapeutic goals and for treatment improvement. Accordingly, many new metal compounds exploiting various transition metals have been prepared and evaluated, and some of them (e.g., gold, silver, copper, ruthenium, and other active metals) turned out to manifest very encouraging antitumor effects. In particular, coinage metals (especially Au and Ag) revealed a greater application potential as they are, on average, less toxic to humans than other transition metals. From a chemical point of view, gold compounds deserve particular attention owing to the unique position of gold in the periodic table, which ultimately leads to a larger electronegativity, a higher electron affinity, and a rich and peculiar redox profile. Several gold compounds, including both gold(III) and gold(I) compounds [i.e., gold(I) carbene, gold sodium thiomalate, gold thiolates, or gold compounds with bipyridyl-type ligands], were thus considered for cancer treatment, and many of them were found to possess remarkable antiproliferative properties *in vitro* against several human cancer cell lines[24].

Several studies have pointed out that gold compounds cause their outstanding cytotoxic effects by taking advantage of multiple molecular and cellular mechanisms. The most credited mechanisms involve inhibition of thiol-containing enzymes, especially thioredoxin reductase (TrxR)[25-27], direct mitochondrial damage[28-31], or alteration of DNA functions[32,33], all of which may contribute importantly to the observed anticancer actions. Although no non-platinum metal compound has been approved so far for clinical use, some gold drug candidates are being actively investigated. A few gold compounds have shown very

promising results in preclinical research[34], and two of them even reached clinical trials.

AURANOFIN AND ITS ANALOGS AS ANTICANCER AGENTS: CHEMICO-BIOLOGICAL ASPECTS AND MECHANISTIC INFERENCES

In the field of gold-based drugs, auranofin (AF) undoubtedly occupies a pivotal position. From the chemical point of view auranofin, i.e., [2,3,4,6-tetra-o-acetyl-l-thio-β-d-glyco-pyrano-sato-S-(triethyl- phosphine)-gold(I)], is a mixed ligand gold(I) complex with a linear geometry having a triethylphosphine molecule and a thioglucose derivative as gold(I) ligands [Figure 1].

Recently, various research groups have found that auranofin[35,36], beyond its known anti-inflammatory actions, also exhibits prominent anticancer, antibacterial, and antiparasitic properties[37-43]. Accordingly, during the past few years, auranofin has attracted a lot of attention in the medicinal chemistry scientific community as a prospective anticancer and anti-infective agent on the ground of drug repurposing strategies. As a matter of fact, AF has entered several different clinical trials as an anticancer, antiviral, or antiparasitic drug (ClinicalTrials.gov Identifier: NCT02089048, NCT01747798, NCT03456700, NCT01419691, NCT02063698, NCT02736968, NCT01737502, NCT02961829, NCT02770378, NCT03975790, and NCT01557348).

Auranofin was initially prepared in the late 1970s and found to manifest remarkable antiarthritic properties. Owing to its favorable pharmacological profile, auranofin was eventually approved for clinical use against rheumatoid arthritis in 1985, although in the absence of a precise understanding of its mode of action.

Moreover, it was found that auranofin was able to inhibit the growth of tumor cells *in vitro* and arrest the growth of an *in vivo* model tumor (leukemia P388) in mice. In fact, Mirabelli *et al.*[44], starting from auranofin and changing systematically the phosphine ligand, the sulfur ligand, or both ligands, obtained 62 distinct Au(I) complexes. These novel complexes were tested both *in vitro*, against B16 melanoma and P388 leukemia, and *in vivo*, against mouse P388 intraperitoneal leukemia, with encouraging results. Mechanistically, it could be ascertained that auranofin behaves as a prodrug capable of releasing its two originals ligands; in any case, it is well documented that the thiosugar ligand is a better leaving group than the phosphine and is the first ligand to be released. The resulting empty coordination position on the gold(I) center becomes available for coordination to biomolecules. Typically, gold compounds with the general formula Et_3PAuX manifest relevant and roughly similar anticancer profiles. This finding implies that the thiosugar ligand is not fundamental for the cytotoxic activity and that the $[Et_3PAu]^+$ moiety is likely to be the true pharmacophore. The role of the X ligand is probably related to the cellular uptake: a more lipophilic nature of the ligand might determine a more favorable pharmacokinetic and biodistribution profile.

The chemical behavior of auranofin and its reactions with biomolecules have been intensely investigated. The reaction of auranofin with biomolecules has now been studied in detail: it could be established that auranofin binds proteins tightly by forming strong coordinative bonds to free cysteine or selenocysteine residues[45,46]. This type of reactivity may well account for its molecular mechanisms; for instance, there is now a general consensus that the tight binding of auranofin to the free selenocysteine group in the active site of TrxR is primarily responsible for its relevant actions at the cellular level, eventually leading to severe intracellular redox dysregulation and associated apoptotic cancer cell death [Figure 2].

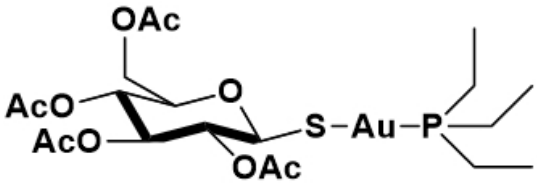

Figure 1. Chemical structure of auranofin.

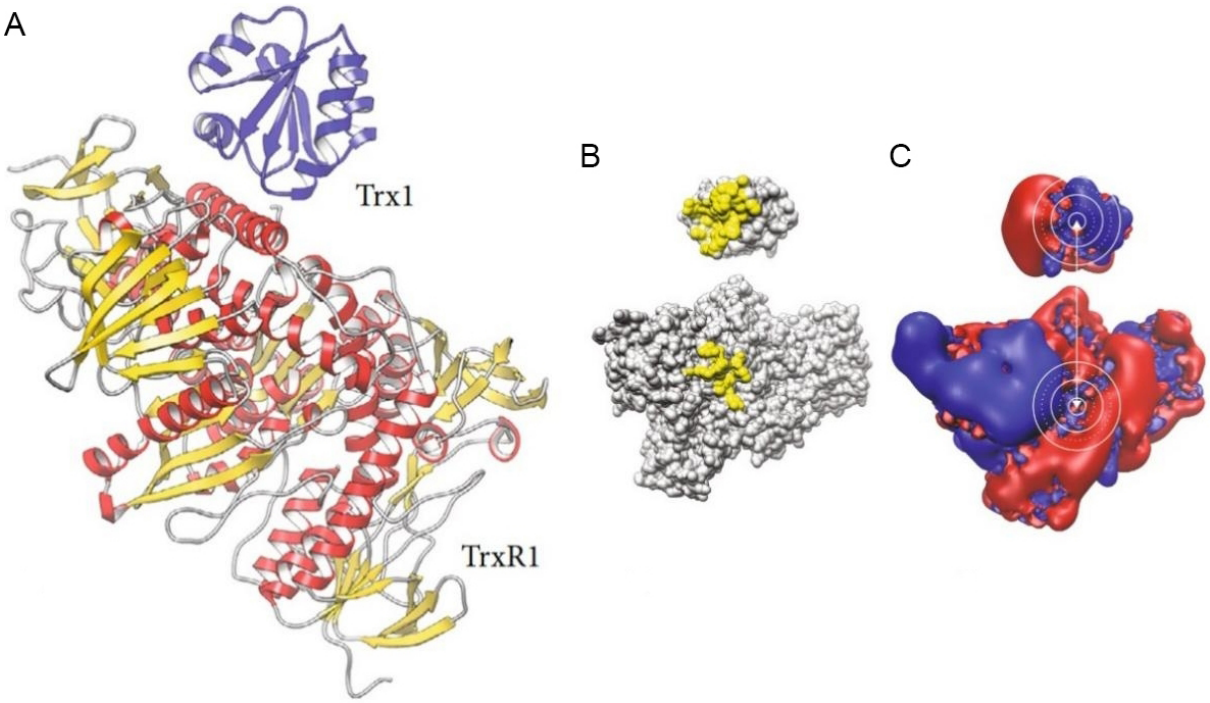

Figure 2. (A) Cartoon representation of the secondary structures of human thioredoxin-1-thioredoxin reductase 1 (Trx1-TrxR1) complex PDB: 3qfa. (B) The contact patches with direct molecular contacts are highlighted in yellow. (C) The isosurfaces of the electrostatic potential are depicted in blue (positive) and red (negative). The active site cysteinyl residues and interaction surfaces in the immediate contact area in both proteins are encircled in white lines. Reproduced and adapted from Hossain *et al.*[47] under the Creative Commons Attribution License 4.0 International (CC BY 4.0).

However, the interpretation of the mechanism of the anticancer actions of auranofin is still controversial and not conclusive. Although thioredoxin reductase inhibition is believed to be a central trait of its mode of action, other likely targets have been proposed and partially validated. As a matter of fact, some interesting proteomics data highlight, upon auranofin treatment, a few differentially expressed proteins belonging to different cellular processes, namely cell redox homeostasis, metabolism, and cell structure. Specifically, the main altered proteins were peroxiredoxins 1 and 6, linked to cell redox balance; triosephosphate isomerase 1, which plays a key role in the glycolysis and gluconeogenesis pathways; ezrin, essential for cell structure and cell migration; and the heterogeneous nuclear ribonucleoprotein H, whose increased cleavage leads to the caspase 3 activation triggering apoptosis[24-48].

In addition, a few recent studies suggested that AF produces important immunomodulatory effects[49]. In particular, it was found that AF induces ICD (immunogenic cell death) in cancer cells as a consequence of endoplasmic reticulum stress and reactive oxygen species (ROS) production. Notably, Freire Boullosa *et al.*[50] showed a significant increase in ICD-related damage-associated molecular patterns

and maturation in dendritic cells following AF treatment in mutant p53 NSCLC *in vitro*. Apparently, these effects are mediated by the immunosuppressive TGF-β cytokine. TGF-β plays a major role in immunosuppression within the tumor microenvironment through the prevention of immune infiltration into tumor tissue and promotion of tumor cell proliferation. The cooperation between AF and anti-PD-L1 therapy in triple-negative breast cancer mouse model further supports the use of AF as an immunomodulating agent[42]. These findings are in accord with some early observations on the immunomodulatory properties of AF[49].

Overall, these arguments demonstrate that auranofin, as already hypothesized for this compound as well as other gold(I) compounds, possesses a multi-target mode of action; indeed, auranofin interacts in cells and blood with several targets, mainly proteins with key functions, involving multiple cellular pathways and altering different biological networks.

AURANOFIN AND ITS ANALOGS SHOW PROMISE FOR COLORECTAL CANCER TREATMENT: *IN VITRO* EVIDENCE

The favorable chemical and pharmacological profile of auranofin as a prospective anticancer agent prompted researchers to synthesize and characterize some auranofin derivatives and assess their action *in vitro* against a few CRC cell lines. In particular, in recent studies, five analogs of auranofin were prepared where the thiosugar ligand was substituted by different anionic ligands (these compounds are depicted in Figure 3)[51,52]. The synthesis of these analogs is rather straightforward and starts from the commercially available Et_3PAuCl. More precisely, the iodide analog can be prepared through a simple chloride-displacing reaction carried out with an excess of potassium iodide[51]; the cyanide derivative can be synthesized reacting the two ionic species $K[Au(CN)_2]$ and $[Au(PEt_3)_2]Cl$ in a biphasic reaction[52]; and the thiocyanate and azido derivatives can be prepared by reacting the Et_3PAuCl species, respectively, with potassium thiocyanate and sodium azide after its activation with silver nitrate[52].

Then, the antiproliferative properties of two of these analogs, i.e., Et_3PAuCl and Et_3PAuI, as well as auranofin itself were comparatively assayed *in vitro* against four representative colorectal cancer lines, i.e., HCT8, HCT116, HT29, and Caco2, and two healthy cell lines, i.e., HDF (human fibroblast, adult) and HEK293 (human embryonic kidney), by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test[51]. As displayed in Table 1, all tested compounds produced very notable cytotoxic effects on all the selected CRC cell lines with half-maximal inhibitory concentration (IC_{50}) values always falling in the 100-700 nM range. Et_3PAuI was slightly less cytotoxic than the other two gold complexes. In line with expectations, the presence of the thiosugar ligand turned out not to be an essential feature for the cytotoxic action. Moreover, by considering the close similarity in the measured IC_{50} values, it can be inferred that the cellular uptake of the three compounds should not be very different. Remarkably, when measuring the cytotoxic effects on two healthy cell lines, HDF human fibroblast cells (adult) and HEK293 human embryonic kidney, no appreciable cytotoxic effects were detected for the three study complexes in the concentration range 0-5000 nM. The latter finding is a good index of selectivity for cancer cells.

Afterwards, since TrxR is reputed to be a primary target for auranofin, the inhibitory potencies of the three gold compounds against this enzyme were analyzed comparatively. The results are summarized in Table 2. Interestingly, the obtained IC_{50} values for TrxR inhibition are roughly consistent with those determined for the cytotoxic effects in CRC cell lines [Table 1]. This probably implies that the observed cytotoxic effects are somehow linked to the ability of these gold complexes to inhibit TrxR. Moreover, the present results confirm that Et_3PAuCl is not only the most potent cytotoxic agent but also the most potent TrxR inhibitor of the series; although the IC_{50} values measured for auranofin and Et_3PAuI are only slightly higher, they still

Table 1. IC_{50} values (nM) determined for Et_3PAuI, AF, and Et_3AuP-Cl (24 h incubation)

Complex	HCT8	HCT116	HT29	Caco2	HDF	HEK293
AF	132 ± 16	180 ± 17	359 ± 35	465 ± 53	> 5000	> 5000
Et_3PAuCl	105 ± 11	154 ± 22	122 ± 15	560 ± 93	> 5000	> 5000
Et_3PAuI	260 ± 28	290 ± 36	318 ± 90	706 ± 232	> 5000	> 5000

The results are reported as the average value for three independent experiments ± standard deviation. Reproduced and adapted with permission from Marzo *et al.*[51]. IC_{50}: Half-maximal inhibitory concentration; AF: auranofin.

Table 2. Thioredoxin reductase activity assay. IC_{50} values (nM) were determined treating 2 U/L of TrxR with aliquots of AF, Et_3 AuPCl, and Et_3PAuI (from 1 ×M to 1 nM)

Complex	IC_{50} (nM)
AF	105 ± 17.3
Et_3AuPCl	51.3 ± 8.5
Et_3PAuI	193 ± 22.2

The results are reported as the average value for three independent experiments ± standard deviation. IC_{50} refers to 50% enzyme inhibition. Reproduced and adapted with permission from Marzo *et al.*[51]. IC_{50}: Half-maximal inhibitory concentration; AF: auranofin.

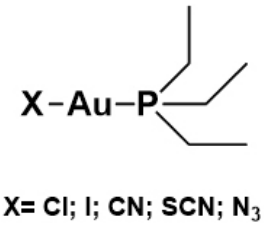

Figure 3. Chemical structures of auranofin analogs bearing different ligands in place of thiosugar moiety.

fall in the nanomolar range.

Conversely, the three auranofin analogs in which the thiosugar was replaced with stronger ligands (i.e., Et_3 PAuCN, $Et_3PAuSCN$, and Et_3PAuN_3) turned out to be completely inactive against HCT116 cell line for concentrations ranging up to 1000 nM. This observation strongly supports the occurrence of a reaction mechanism in which the anionic groups have to be displaced from the nucleophilic active site of TrxR (the SEC-CYS motif) to observe the pharmacological activity of the $[Au(PEt_3)]^+$ moiety[52]. In conclusion, the results of this study demonstrated that auranofin and its chloride, and the iodide analogs manifest potent cytotoxic effects *in vitro* against four selected CRC lines with the measured IC_{50} values always falling in the nanomolar range and no apparent cytotoxic effect on human fibroblast cell line and human embryonic kidney cells up to a 5 μM concentration. The TrxR activity assay revealed that both Et_3PAuCl and Et_3PAuI retain the potent inhibitory action of auranofin (nanomolar range), being consistent with their observed cytotoxic effects. Overall, these findings are consistent with the concept that TrxR remains the most probable and most relevant biomolecular target for these gold compounds. Moreover, these results, although obtained *in vitro* on cell cultures, support the idea that auranofin and its analogs are optimal drug candidates for further testing against more advanced and sophisticated CRC models. It should also be noted that the DNA-independent mode of action of AF and some of its analogs is a key aspect determining the high cytotoxicity toward *in vitro* CRC models. As an example, AF, Et_3PAuCl, and Et_3PAuI [Table 1] have been reported to exert anticancer effects significantly greater than cisplatin and even oxaliplatin on

representative colorectal cancer cell lines [Table 3][51,52].

Based on the above considerations, it could be inferred that the ability of auranofin and some of its analogs to exert a greater anticancer activity in CRC lines than cisplatin and oxaliplatin might depend on the different mechanisms underlying the pharmacological effects. In fact, in contrast to oxaliplatin[55], auranofin does not efficiently bind DNA, thus being its activity substantially unaffected by MMR, p53, and nucleotide excision repair functions. In fact, gold-based drugs produce the desired anticancer effects mainly through a DNA-independent mode, i.e., targeting specific enzymes such as the Trx system[51,52].

NEW PERSPECTIVES IN THE USE OF AURANOFIN AND ITS ANALOGS: ENCAPSULATION OF GOLD COMPOUNDS IN BIOCOMPATIBLE NANOPARTICLES

As stated above, Et_3PAuCl is an auranofin derivative exhibiting very attractive biological and pharmacological properties. Similar to auranofin, Et_3PAuCl possesses potent cytotoxic properties *in vitro* toward numerous cancer cell lines, thus being a promising anticancer drug candidate. In this frame, some investigators wondered whether Et_3PAuCl encapsulation might lead to a better pharmacological profile, considering the expected reduction of unwanted side-reactions that are mainly responsible for the adverse effects and for drug inactivation. A reasonable option to achieve this goal consists in using biocompatible nanoparticles as nanocarriers to protect the gold complex from the biological environment. To achieve this goal, Menconi *et al.*[43] exploited organic polyethylene glycol-poly lactic acid-co-glycolic acid (PLGA-PEG)-based nanoparticles of intermediate size, which could host a certain number of metallodrug's copies into their hydrophobic core [Figure 4]. Et_3PAuCl was encapsulated in these biocompatible PLGA-PEG nanoparticles, and the new formulation was evaluated in colorectal HCT116 cancer cells in comparison to free Et_3PAuCl.

Notably, the encapsulated Et_3PAuCl mostly retains the cellular actions of the free complex and causes even larger cytotoxic effects in CRC cells, through apoptosis and autophagy. Moreover, a large inhibition of two crucial signaling pathways, namely extracellular signal-regulated kinase (ERK) and protein kinase B (AKT), by the encapsulated form of Et_3PAuCl, was clearly evidenced by the fact that this inhibition was not found in cells treated with the free drug. Overall, these results point out that encapsulation of Et_3PAuCl in PLGA-PEG nanoparticles does not significantly affect the antiproliferative properties of this gold complex. However, some changes in the biological effects of the studied gold complex could be detected, which were specifically evidenced by the differential effects produced on the ERK and AKT signaling pathways. It would be of interest to extend such experimentation to appropriate *in vivo* models of CRC.

NEW PERSPECTIVES IN THE USE OF AURANOFIN AND ITS ANALOGS: THE ROLE OF COMBINATION THERAPIES

Another valuable strategy in the use of gold compounds as anti-colorectal cancer agents is offered by the exploitation of so-called combination therapies. It is a common practice in cancer pharmacology to use anticancer drugs in association. Cocktails of drugs instead of single drugs are indeed very popular in current anticancer medical treatments for various reasons: (1) the application of lower concentrations of intrinsically toxic drugs with a narrow therapeutic index; (2) the opportunity to achieve a considerable synergism; and (3) the effective chance of reducing resistance insurgency. Combination therapy may also consist of the combination of an anticancer drug with a common non-cytotoxic drug that has nonetheless the important potential to modulate/enhance the cytotoxic effect of the first drug. This type of strategy was applied very recently by Han *et al.*[56] to auranofin for the treatment of CRC. In detail, these authors performed a high-throughput screening of a library of 1280 FDA-approved clinical drugs in the search for compounds that might enhance the anticancer activity of auranofin *in vitro*. Surprisingly, they found that

Table 3. The IC_{50} values (×M) determined for cisplatin and oxaliplatin (24 h incubation) against HT29 and HCT116 lines are also included as reference

Complex	HCT116	HT29
Cisplatin	21.96 ± 1.11	16.39 ± 1.10
Oxaliplatin	49.2 ± 0.9	19.7 ± 1.2

Reproduced and adapted with permission from Marzo *et al.*[53] and Cirri *et al.*[54]. IC_{50}: Half-maximal inhibitory concentration.

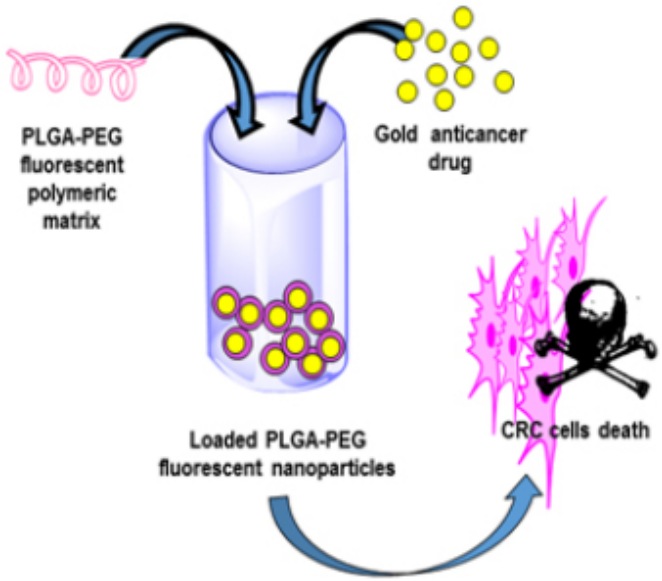

Figure 4. Schematic representation of the Et_3PAuCl-loaded NPs tested against colorectal cancer models. Reproduced and adapted from Menconi *et al.*[43] under the Creative Commons Attribution License 4.0 International (CC BY 4.0).

the anti-inflammatory drug celecoxib (CE), a cyclooxygenase 2 inhibitor, strongly potentiated the anticancer activity of AF[56].

Notably, the promising *in vitro* results obtained for the AF + CE association were later supported by very encouraging *in vivo* results, as displayed in Figure 5. Since AF and CE are FDA-approved drugs that are used in the clinic, it is quite straightforward to translate the results of this study into an immediate clinical cancer treatment.

Mechanistically, the AF/CE combination induced severe oxidative stress, resulting in ROS-mediated hexokinase inhibition and disruption of mitochondrial redox homeostasis. Overall, these effects eventually caused a significant decrease of ATP generation. The CE-induced ROS increase together with AF-mediated inhibition of thioredoxin reductase determined a large shift of Trx2 to its oxidized form, producing a degradation of MT-CO2 (mitochondrially-encoded cytochrome C oxidase II) and a dysfunction of the electron transport chain (see Figure 6).

CONCLUSIONS

CRC is the second most deadly cancer worldwide. Medical treatments for CRC are still largely insufficient and rarely curative; it follows that the inoperable metastatic disease results in most cases in patient's death[57]. Metal-based drugs may play a significant and growing role in the therapeutics of CRC; oxaliplatin

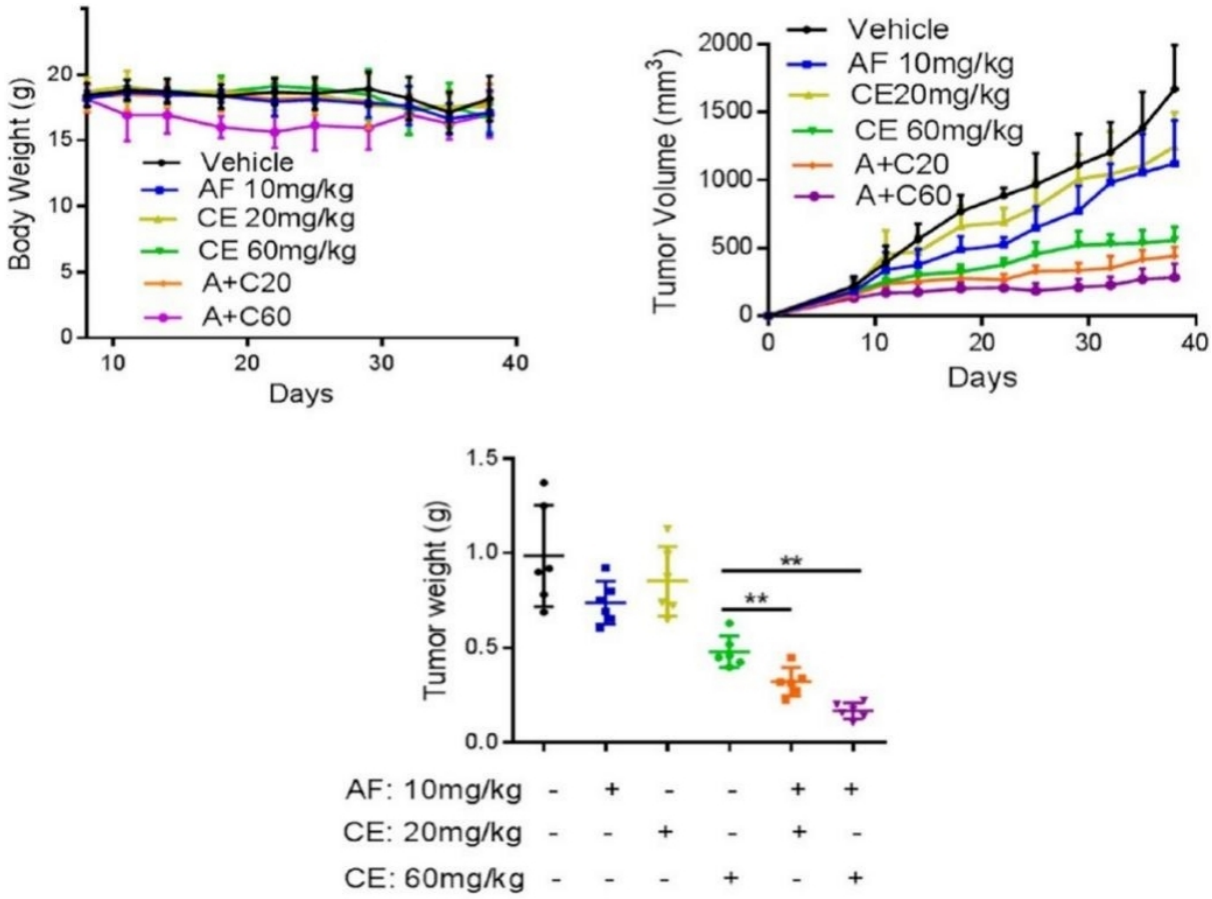

Figure 5. The effect of the combination of auranofin with celecoxib in mice. Athymic nude mice bearing DLD-1 xenografts were treated with the following drugs via oral injection (P.O.): olive oil (vehicle), AF 10 mg/kg, CE 20 mg/kg, CE 60 mg/kg, AF 10 mg/kg + CE 20 mg/kg (A + C20), and AF 10 mg/kg + CE 60 mg/kg (A + C60). Eight days after inoculation, the tumor size and body weight of mice from each group (six mice per group) were measured two times per week. The three panels show the body weight, tumor volume, and tumor weight during the treatment. Reproduced and adapted from Han *et al.*[56] under the Creative Commons Attribution License 4.0 International (CC BY 4.0). AF: Auranofin; CE: celecoxib.

is already employed in the treatment of this disease, but its use is often limited by the insurgence of platinum resistance. Gold compounds are promising experimental anticancer drugs and might offer a valuable alternative to platinum drugs by overcoming resistance to Pt drugs[58]. Among the existing medicinal gold compounds, auranofin and its analogs - given the ease of their repurposing - may be the most appropriate and obvious drug candidates for CRC[59]. Auranofin has indeed manifested relevant anticancer actions and already entered clinical trials for other types of cancer, in particular ovarian cancer and various hematological malignancies. Surprisingly, auranofin has been scarcely tested so far for CRC treatment. However, just a few years ago, it was demonstrated that AF is very effective *in vitro* against four representative CRC lines while being far less toxic for healthy cells, thus showing some degree of selectivity. Moreover, a few studies in the recent literature suggest new valuable strategies to improve the pharmacological profiles of AF and its analogs. We refer specifically to a couple of studies that delineate feasible strategies for therapeutic intervention[43,56]. In a first study, encapsulation of Et_3PAuCl in PLGA nanoparticles proved to bring about some favorable pharmacological effects such as retention of the cytotoxic activity, attenuation of the general reactivity of the gold center, and expected reduction of the drug's adverse effects. Alternatively, a second study showed that the anticancer action of AF in CRC may be greatly potentiated through appropriate drug combinations. In fact, a systematic screening procedure

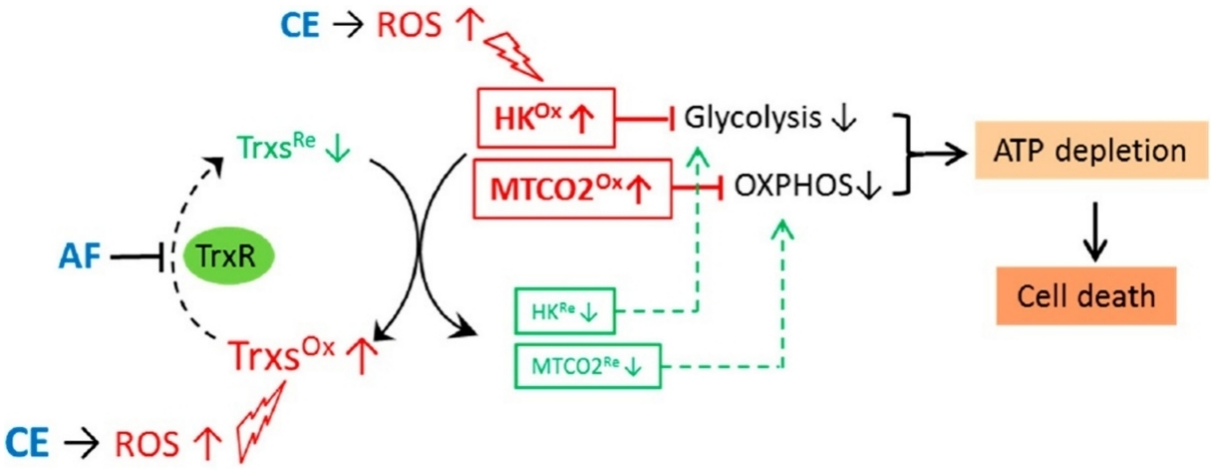

Figure 6. Synergy between auranofin and celecoxib against colon cancer *in vitro* and *in vivo* through a novel redox-mediated mechanism. CE induces ROS increase, which in turn causes oxidation of proteins (Trxs, HK, and MTCO2). AF inhibits TrxR, and thus keeps Trxs in oxidized form, which cannot reduce/repair the oxidized proteins (HK and MTCO2), leading to inhibition of both glycolysis and mitorespiration, ATP depletion, and cell death. Reproduced and adapted from Han *et al.*[56] under the Creative Commons Attribution License 4.0 International (CC BY 4.0). CE: Celecoxib; ROS: reactive oxygen species; HK: hexokinase.

applied to a large library of 1280 FDA-approved drugs revealed a strong synergism between auranofin and the anti-inflammatory drug celecoxib. This synergism was well documented both *in vitro* and *in vivo*. In view of these initial yet very encouraging results, we propose that the testing of auranofin and its analogs toward suitable CRC models is further expanded and encouraged taking advantage of new pharmaceutical formulations and appropriate drug combinations. Additionally, extensive *in vivo* testing of these gold compounds against suitable animal models of CRC would be highly desirable at this stage to reinforce and validate the concepts presented here.

DECLARATIONS

Authors' contributions

Conception and writing of the manuscript: Massai L, Cirri D, Marzo T, Messori L
All the authors approved the final version of the paper.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Original Article

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Efficacy of ICIs on patients with oncogene-driven non-small cell lung cancer: a retrospective study

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Abstract

Aim: The objective of our study was to assess the efficacy of immune checkpoint inhibitors (ICIs) on patients with non-small-cell lung cancer (NSCLC) harboring oncogenic alterations.

Methods: We retrospectively enrolled patients with advanced non-squamous NSCLC who were treated with anti-PD-1-based monotherapy or combined immunotherapy. Major characteristics including PD-L1 expression, treatment, and survival were analyzed.

Results: In total, 309 non-squamous NSCLC patients with a median age of 61 years (range 20-88 years) including 70.9% male were retrospectively enrolled. The molecular alterations involved epidermal growth factor receptor (EGFR) (n = 81), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (n = 31), anaplastic lymphoma kinase (ALK) (n = 1), human epidermal growth factor receptor 2 (HER2) (n = 12), V-raf murine sarcoma viral oncogene homolog (BRAF) (n = 2), rearranged during transfection (n = 4), and c-ros oncogene 1 (ROS1) (n = 3). In the EGFR subset, the ORR was 30.9% (n = 81) and PFS was significantly shorter than WT group (median PFS: 5.7 months *vs.* 7.1 months; P = 0.0061). In subgroup analyses, ICI combined therapy was significantly correlated with a longer PFS compared with ICI monotherapy (median PFS: 7.7 months *vs.* 4.7 months; P = 0.0112). In KRAS patients, ORR was 51.6% (n = 31). No significant difference was found in subgroup analyses. The ORR and PFS were 16.7%

(*n* = 12) and 28.6% (*n* = 7), 7.8 months and 9.0 months for HER2 and EGFR Exon20 insertion patients, respectively. Three ROS1 patients were enrolled with a PFS of 16.0, 34.2, and 45.0 months individually, and one ALK patient with PFS of 4.4 months was identified. No response was found in two BRAF patients.

Conclusion: ICI-based combination therapy can bring benefit to patients with EGFR-mutant NSCLC. ICI-based combination therapy could be considered for patients with ROS1 rearrangement, HER2 mutation and EGFR Exon20 insertion NSCLC.

Keywords: Non-squamous NSCLC, driver mutations, immune checkpoint inhibitor

INTRODUCTION

Lung cancer is the most common cancer worldwide, in terms of both incidence and mortality[1]. Over the past decades, great advancements have been achieved, which are attributed to the understanding of tumor biology and the molecular mechanism of tumor progression. The use of small molecule tyrosine kinase inhibitors (TKIs) has dramatically improved the prognosis of patients with specific genomic aberrations[2-5]. However, despite the high response of TKIs, acquired resistance inevitably occurs and limits the long-term benefits[6]. Once this happens, the subsequent anti-tumor treatment is limited.

Immune checkpoint inhibitors (ICIs), specifically those targeting PD-1 or programmed death-ligand 1 (PD-L1), have rapidly transformed the treatment paradigm for non-small cell lung cancer (NSCLC). For driver-negative NSCLC, ICIs are now the cornerstone of first-line therapy[7]. However, whether ICIs alone or in combination with other therapies would bring benefit to those with driver mutations is still to be elucidated. Gainor *et al.*[8] reported poor response of ICI monotherapy in patients with epidermal growth factor receptor (EGFR) mutations or anaplastic lymphoma kinase (ALK) rearrangements. In the phase II ATLANTIC study, durvalumab showed activity in driver-positive NSCLC according to final OS analysis[9]. Therefore, continued research is required to explore the optimal use of ICI therapy in patients with driver mutations to improve outcomes of this cohort.

In our study, we retrospectively analyzed patients with locally advanced or metastatic non-squamous NSCLC that were treated with anti-PD-1 based mono- or combined-immunotherapy in Shanghai Pulmonary Hospital to assess the efficacy of ICIs on patients with driver-positive NSCLC.

METHODS

Study population

Patients with locally advanced or metastatic non-squamous non-small cell lung cancer from July 2015 to July 2020 in Shanghai Pulmonary Hospital were retrospectively enrolled. Inclusion criteria included the following: (1) a pathologic diagnosis of non-squamous non-small cell lung cancer; (2) testing data (either direct sequencing or NGS on validated platforms) for EGFR, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), ALK, human epidermal growth factor receptor 2 (HER2), V-raf murine sarcoma viral oncogene homolog (BRAF), rearranged during transfection (RET), and c-ros oncogene 1 (ROS1); and (3) anti-PD-1-based monotherapy or combined immunotherapy as first-line or posterior-line therapy. Patients treated with fewer than two circles of immunotherapies and had no available complete medical records were excluded. The patient screening process is shown in Figure 1. We reviewed the medical records and abstracted the following patient characteristics: age, gender, Eastern Cooperative Oncology Group Performance Status (ECOG-PS), smoking history, histological type, clinical stage, mutation type, PD-L1 expression, details of treatment, and survival.

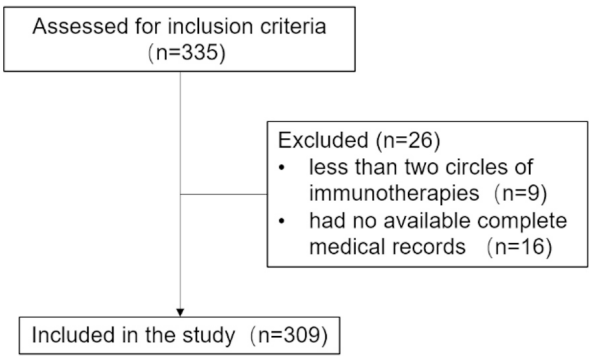

Figure 1. Flow chart of patient screening.

PD-L1 analysis

PD-L1 immunohistochemistry was performed in Department of Pathology, Shanghai Pulmonary Hospital according to routine procedure. The antibody used was PD-L1 22C3 pharmDx (Dako, Carpinteria, CA, USA). PD-L1 positivity was defined as tumor proportion score cutoff of 1%.

Statistical analysis

Patient characteristics are expressed as median and range for continuous variables and as frequencies and percentages for categorical variables. Progression-free survival (PFS) was measured from the date of first administration of PD-1 inhibitor treatment to disease progression or death due to any cause or last follow-up. Response Evaluation Criteria in Solid Tumors guidelines 1.1 (RECIST 1.1) was used to assess tumor response. The difference of response rate among different groups was calculated using Kruskal-Wallis test. Survival data were estimated using the Kaplan-Meier method and compared using the log-rank test in the overall cohort and subgroups. The data were analyzed using GraphPad Prism 8.0 and SPSS Statistics 20. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

In this study, 309 non-squamous non-small cell lung cancer patients treated with anti-PD-1 based monotherapy or combined immunotherapy in Shanghai Pulmonary Hospital were identified. The median age was 61 years (range 20-88 years), 70.9% were male (219/309), and 55.7% (112/309) were never smokers. The majority of the patients had adenocarcinoma histology (98.4%, 304/309) and ECOG-PS of 0-1 at the start of immunotherapy (83.5%, 258/309). Detailed characteristics are shown in Table 1. In total, 182 received anti-PD-1 combined with chemotherapy (pemetrexed combined with carboplatin 85.7%, 156/182; paclitaxel-based chemotherapy 14.3%, 26/182), 42 received anti-PD-1 combined with antiangiogenic therapy (apatinib 90.5%, 38/42; bevacizumab 9.5%, 4/42). None of the enrolled patients received a combination of ICIs, chemotherapy, and antiangiogenic therapy. Among the whole cohort, 134 had genetic alteration, which involved EGFR (n = 81), KRAS (n = 31), ALK (n = 1), HER2 (n = 12), BRAF (n = 2), RET (n = 4), and ROS1 (n = 3). Among the 81 EGFR mutation patients, 42 had exon 19 deletions, 29 had exon20 L858R, and 10 had other mutations; 14 were harboring T790M mutation at the same time and 67 were not. The details of each subgroup are shown in Table 1.

PD-L1 expression

PD-L1 expression status was available for 132 patients, of whom 54.5% (72/132) had less than 1% PD-L1 expression, 31.1% (41/72) had PD-L1 expression of 1%-49%, and 14.4% (19/132) had PD-L1 expression more than 50% [Figure 2A]. Looking into each subgroup, patients with EGFR mutation seemed to have a

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Table 1. Characteristics of patients according to molecular alterations

	All cases	EGFR			KRAS		ALK	HER2	BRAF	RET	ROS1
		19del	20 L858R	Other	G12C	G12D	EML4-ALK	20ins	V600E	RET-KIF5B	CD74-ROS1
	n = 309	*n* = 42	*n* = 29	*n* = 10	*n* = 30	*n* = 1	*n* = 1	*n* = 12	*n* = 2	*n* = 4	*n* = 3
Age	61 (20-88)	61 (20-72)	60 (40-75)	57 (38-73)	61 (49-75)	71	62	59 (45-68)	49, 61	49 (26-61)	41 (41-64)
Gender											
Male	219 (70.9%)	24 (57.1%)	18 (62.1%)	7 (70%)	27 (90%)	1 (100%)	1 (100%)	7 (58.3%)	2 (100%)	2 (50%)	1 (33.3%)
Female	90 (29.1%)	18 (42.9%)	11 (37.9%)	3 (30%)	3 (10%)	0 (0%)	0 (0%)	5 (41.7%)	0 (0%)	2 (50%)	2 (66.7%)
ECOG performance status											
0-1	258 (83.5%)	36 (85.7%)	24 (82.8%)	7 (70%)	26 (86.7%)	1 (100%)	0 (0%)	11 (91.7%)	0 (0%)	3 (75%)	3 (100%)
≥ 2	51 (16.5%)	6 (14.3%)	5 (17.2%)	3 (30%)	4 (13.3%)	0 (0%)	1 (100%)	1 (8.3%)	2 (100%)	1 (25%)	0 (0%)
Smoking history											
Current or former	137 (44.3%)	27 (64.3%)	22 (75.9%)	7 (70%)	11 (36.7%)	1 (100%)	0 (0%)	4 (33.3%)	1 (50%)	0 (0%)	2 (66.7%)
Never	172 (55.7%)	15 (35.7%)	7 (24.1%)	3 (30%)	19 (63.3%)	0 (0%)	1 (100%)	8 (66.7%)	1 (50%)	4 (100%)	1 (33.3%)
Histological type											
Adenocarcinoma	304 (98.4%)	41 (97.6%)	29 (100%)	10 (100%)	30 (100%)	1 (100%)	1 (100%)	11 (91.7%)	2 (100%)	4 (100%)	3 (100%)
NOS	5 (0.6%)	1 (2.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)
Clinical Stage											
Stage IIIB	2 (0.7%)	0 (0%)	1 (3.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Stage IIIC	5 (1.6%)	1 (2.4%)	1 (3.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Stage IV	302 (97.7%)	41 (97.6%)	27 (93.1%)	10 (100%)	30 (100%)	1 (100%)	1 (100%)	12 (100%)	2 (100%)	4 (100%)	3 (100%)
PD-L1 expression											
< 1%	72 (23.3%)	11 (26.2%)	5 (17.2%)	2 (20%)	5 (16.7%)	0 (0%)	0 (0%)	5 (41.7%)	0 (0%)	3 (75%)	2 (66.7%)
1%-49%	41 (13.3%)	2 (4.8%)	2 (6.9%)	0 (0%)	5 (16.7%)	0 (0%)	0 (0%)	2 (16.7%)	0 (0%)	0 (0%)	1 (33.3%)
≥ 50%	19 (6.1%)	3 (7.1%)	1 (3.4%)	0 (0%)	5 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)	0 (0%)
Unknown	177 (57.3%)	26 (61.9%)	21 (72.4%)	8 (80%)	15 (50%)	1 (100%)	1 (100%)	5 (41.7%)	2 (100%)	0 (0%)	0 (0%)
Line of ICIs											
1	106 (34.3%)	1 (2.4%)	1 (3.4%)	4 (40%)	15 (50%)	0 (0%)	0 (0%)	6 (50%)	0 (0%)	2 (50%)	3 (100%)
2	127 (41.1%)	26 (21.9%)	17 (58.6%)	2 (20%)	12 (40%)	1 (100%)	0 (0%)	3 (25%)	0 (0%)	2 (50%)	0 (0%)
≥ 3	76 (24.6%)	15 (35.7%)	11 (37.9%)	4 (40%)	3 (10%)	0 (0%)	1 (100%)	3 (25%)	2 (100%)	0 (0%)	0 (0%)
Treatment modality											
Anti-PD-1 monotherapy	85 (27.5%)	2 (4.8%)	5 (17.2%)	1 (10%)	9 (30%)	1 (100%)	1 (100%)	4 (33.3%)	2 (100%)	1 (25%)	0 (0%)
Anti-PD-1 plus chemotherapy	182 (58.9%)	39 (92.9%)	22 (75.9%)	7 (70%)	17 (56.7%)	0 (0%)	0 (0%)	7 (58.3%)	0 (0%)	3 (75%)	3 (100%)
Anti-PD-1 plus antiangiogenic therapy	42 (13.6%)	1 (2.4%)	2 (6.9%)	2 (20%)	4 (13.3%)	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)

ICIs: Immune checkpoint inhibitors; NOS: not otherwise specified; EGFR: epidermal growth factor receptor; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; ALK: anaplastic lymphoma kinase; HER2: human epidermal growth factor receptor 2; BRAF: V-raf murine sarcoma viral oncogene homolog; RET: rearranged during transfection; ROS1: c-ros oncogene 1; ECOG: Eastern Cooperative Oncology Group.

low PD-L1 expression (26 patients: 69.2%, 18/26 with PD-L1 < 1%, 15.4%, 4/26 with PD-L1 1%-49%, and 15.4%, 4/26 with PD-L1 ≥ 50%) [Figure 2B]. In the KRAS subgroup, the percentages of PD-L1 < 1%, 1%-49%, and ≥ 50% were all 33.3% (5/15) [Figure 2C]. Of the seven HER2 cases, 71.4% (5/7) had < 1% PD-L1 expression, while 28.6% (2/7) had a PD-L1 expression of 1%-49% [Figure 2D].

Response rate

Based on RECIST 1.1, the ORR of the patients with wild type (WT) was 34.9% and DOR was 50.3% (n = 175), while, in the EGFR subset, the ORR was 30.9% and DOR was 77.8% (n = 81). For KRAS patients, ORR was 51.6% and DOR was 83.9% (n = 31). The response rates in both the EGFR group and the KRAS group were statistically different from the WT group (P = 0.029 and P = 0.004, respectively) [Figure 3A]. In EGFR patients, the ICI combination therapy subset seemed to have better response rate compared to those who received ICI monotherapy (P = 0.020) [Figure 3B]. However, the difference was not found in KRAS patients [Figure 3C]. In 12 HER2 patients, ORR was 16.7% and DCR was 91.7%. While ORR was 28.6% and DCR was 85.7% in EGFR Exon20 insertion patients [Figure 3A].

Progression-free survival

EGFR

We investigated the outcomes of ICIs on patients with EGFR mutations. PFS of the EGFR subset was significantly shorter than that of the WT group (median PFS: 5.7 months *vs.* 7.1 months; P = 0.0061) [Figure 4A]. Regarding PD-L1 expression, PFS was not significantly different (P = 0.3721) [Figure 4B]. ICI combined therapy was significantly correlated with a longer PFS compared with ICI monotherapy (median PFS: 7.7 months *vs.* 4.7 months; P = 0.0112) [Figure 4C]. PFS was 5.5 months in L858R, 5.9 months in 19del, and 9.0 months in Exon20 insertion and other mutations, but the difference was not statistically significant among the three groups (P = 0.3411) [Figure 4D]. There was no difference in PFS between patients with or without T790M mutation (median PFS: 5.6 months *vs.* 5.9 months; P = 0.8381) [Figure 4E].

KRAS

KRAS was not associated with a benefit on PFS compared to those harboring no gene alteration (median PFS: 11.0 months *vs.* 7.1 months; P = 0.5714) [Figure 5A]. In subgroup analyses, PD-L1 positive patients seemed to have longer PFS than PD-L1 negative ones, but the difference was not statistically significant (median PFS: 15.8 months *vs.* 5.6 months; P = 0.0670) [Figure 5B]. ICI combined therapy had no advantage in PFS compared with ICI monotherapy (median PFS: 12 months *vs.* 7.25 months; P = 0.5714) [Figure 5C].

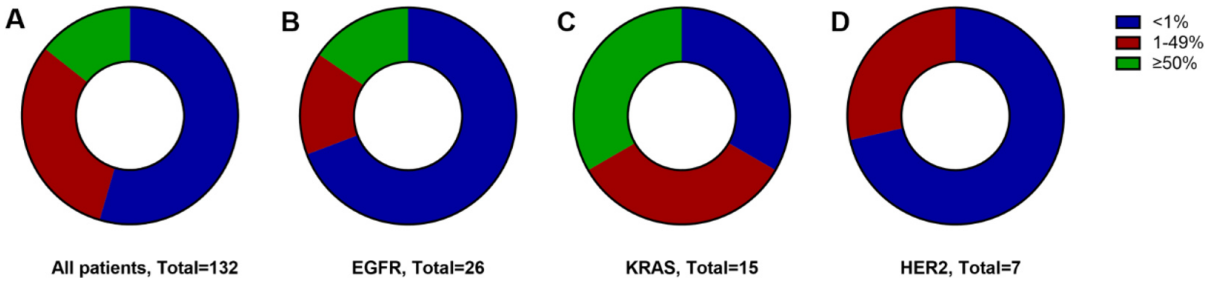

Figure 2. Categorical distribution of tumor PD-L1 expression: the whole cohort (A); EGFR-mutant NSCLC tumors (B); KRAS-mutant NSCLC tumors (C); and HER2-mutant NSCLC tumors (D). EGFR: Epidermal growth factor receptor; NSCLC: non-small-cell lung cancer; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; HER2: human epidermal growth factor receptor 2.

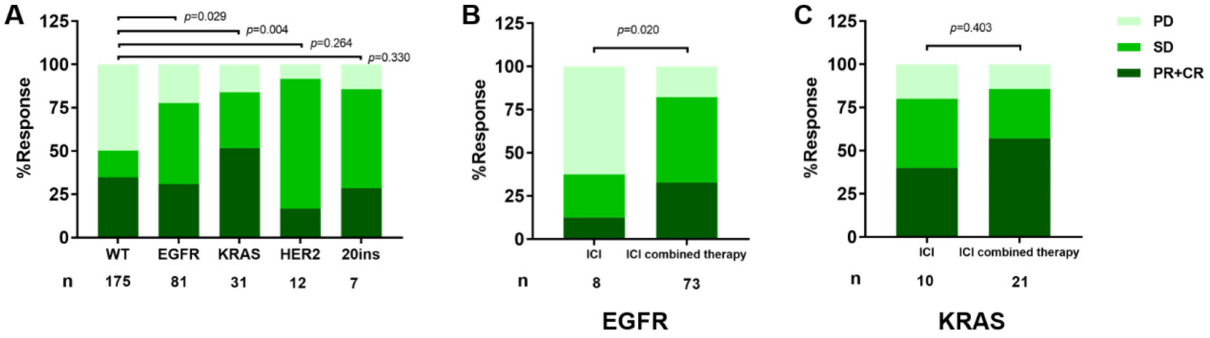

Figure 3. Response to ICIs according to Response Evaluation Criteria in Solid Tumors guidelines 1.1 (RECIST 1.1): (A) response to ICIs across molecular alterations; (B) response rate by treatment modality in EGFR patients; and (C) response rate by treatment modality in EGFR patients. ICIs: Immune checkpoint blockades; PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response; WT: wild type. EGFR: epidermal growth factor receptor; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog.

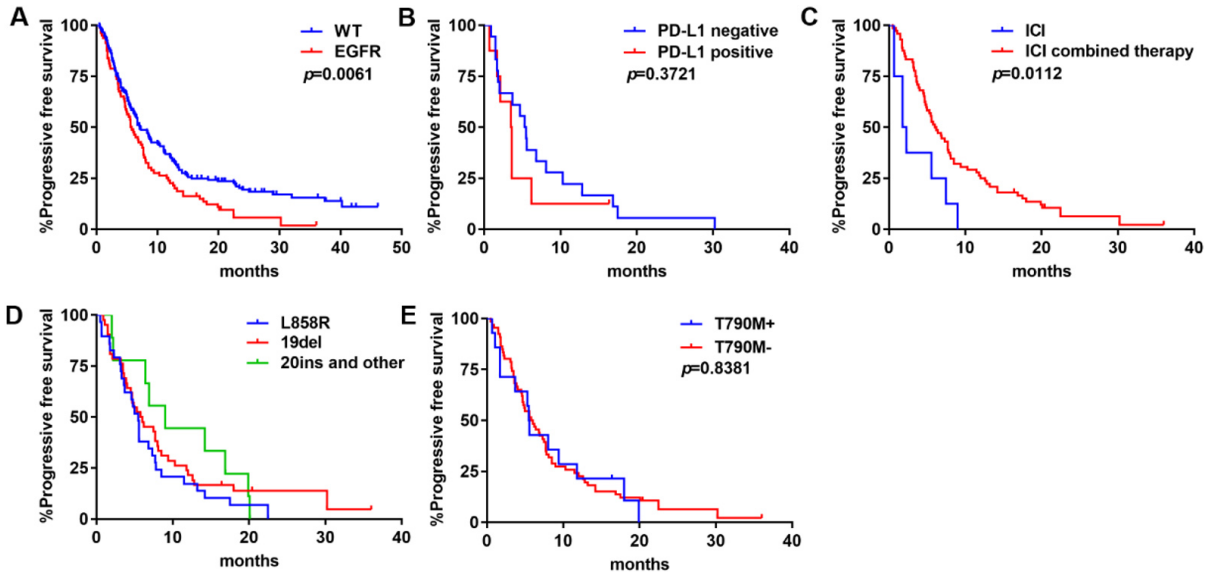

Figure 4. PFS of ICIs in EGFR-mutant NSCLC: (A) PFS in tumors with WT NSCLC or EGFR-mutant NSCLC; (B) PFS by PD-L1 expression levels; (C) PFS by treatment modality; (D) PFS by mutation type; and (E) PFS by T790M mutation status. ICIs: Immune checkpoint blockades; PFS: progression-free survival; WT: wild type; EGFR: epidermal growth factor receptor; NSCLC: non-small-cell lung cancer.

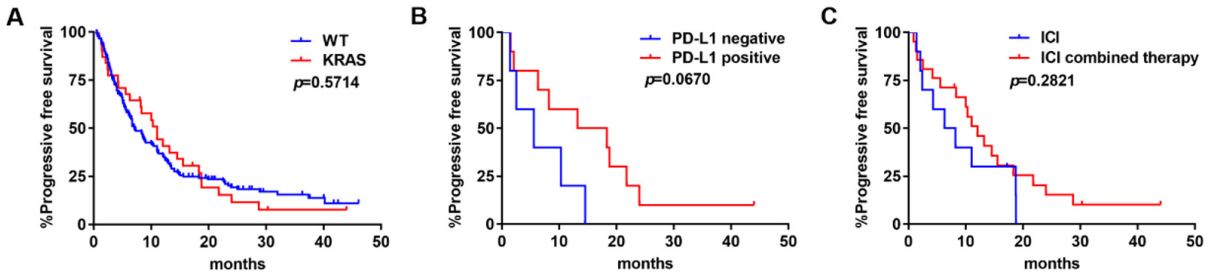

Figure 5. PFS of ICIs in KRAS-mutant NSCLC: (A) PFS in tumors with WT NSCLC or KRAS-mutant NSCLC; (B) PFS by PD-L1 expression levels; and (C) PFS by treatment modality. ICIs: Immune checkpoint blockades; PFS: progression-free survival; WT: wild type; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; NSCLC: non-small-cell lung cancer.

Other molecular alterations

All three patients with ROS1 rearrangements and ECOG 1 who received ICIs combined with chemotherapy as first-line treatment had response of PR. The PFS was 16.0, 34.2, and 45.0 months, respectively. The response of the patient with the longest PFS (45 months) was ongoing [Figure 6]. Four patients with RET rearrangements had PFS of 1.5, 1.8, 4.3, and 9.8 months. Three of them had ECOG 1 and one had ECOG 2. They received first-line (n = 2) and second-line (n = 2) ICI monotherapy (n = 1) or ICIs combined with chemotherapy (n = 3). No response was found in two BRAF patients, who received after second-line ICI monotherapy. Most HER2 patients had ECOG 1 (n = 11). Seven of them received ICIs combined with chemotherapy, four received ICI monotherapy, and one received ICIs combined with antiangiogenic therapy. ICIs as first-line therapy were observed in six patients, as second-line in three patients, and after second-line in three patients. The median PFS was 7.8 months (range 1.0-26.9 months). ALK rearrangements was identified in only one patient, whose PFS was 4.4 months, with ECOG 3, who received ICI monotherapy as after second-line treatment. We also enrolled seven patients with EGFR Exon20 insertion. The median PFS was nine months (range 2.2-20.1 months). They received ICIs combined with chemotherapy (n = 5) or antiangiogenic therapy (n = 1) or ICI monotherapy (n = 1), as first-line (n = 4), second-line (n = 1), or after second-line treatment (n = 2), with ECOG 1 (n = 5), 2 (n = 1), or 3 (n = 1).

DISCUSSION

Targeted therapies against oncogene-driven NSCLC, including EGFR[2], BRAF[10], and HER2 mutations[11] or ALK[3], ROS1[5], and RET[12] rearrangements, have been proved to improve outcomes, and they form the standard first-line treatment in patients with advanced disease. However, progression inevitably occurs, and chemotherapy is currently the main subsequent treatment after TKI resistance. Considering the remarkable long-term benefits of ICI treatments, scientists have made lots of efforts to integrate ICIs into the treatment course of patients with oncogene-driven NSCLC. Mazieres *et al.*[13] explored the activity of ICIs across NSCLC harboring oncogenic alterations and found driver-positive NSCLC exhibited poor response to ICI monotherapy. Hastings *et al.*[14] reported more favorable outcomes of exon 21 mutations compared with exon 19 deletions in NSCLC treated with immunotherapy. Until now, the results on this topic are controversial.

In EGFR-driven NSCLC, initial clinical results indicate that ICIs have no clinical benefits. Meta-analysis[15,16] of EGFR-mutated patients in Keynote 010, CheckMate 057, OAK, and POPLAR studies showed ICIs have poorer outcomes in cohorts with EGFR mutations compared to chemotherapy. We found similar findings in our research that patients with EGFR mutations had poor response to ICIs. This may be explained by the immunosuppressive and uninflamed tumor microenvironment (TME) and low tumor mutational burden (TMB), therefore being less immunogenic in the context of oncogenic addiction[17,18]. Besides the effect of

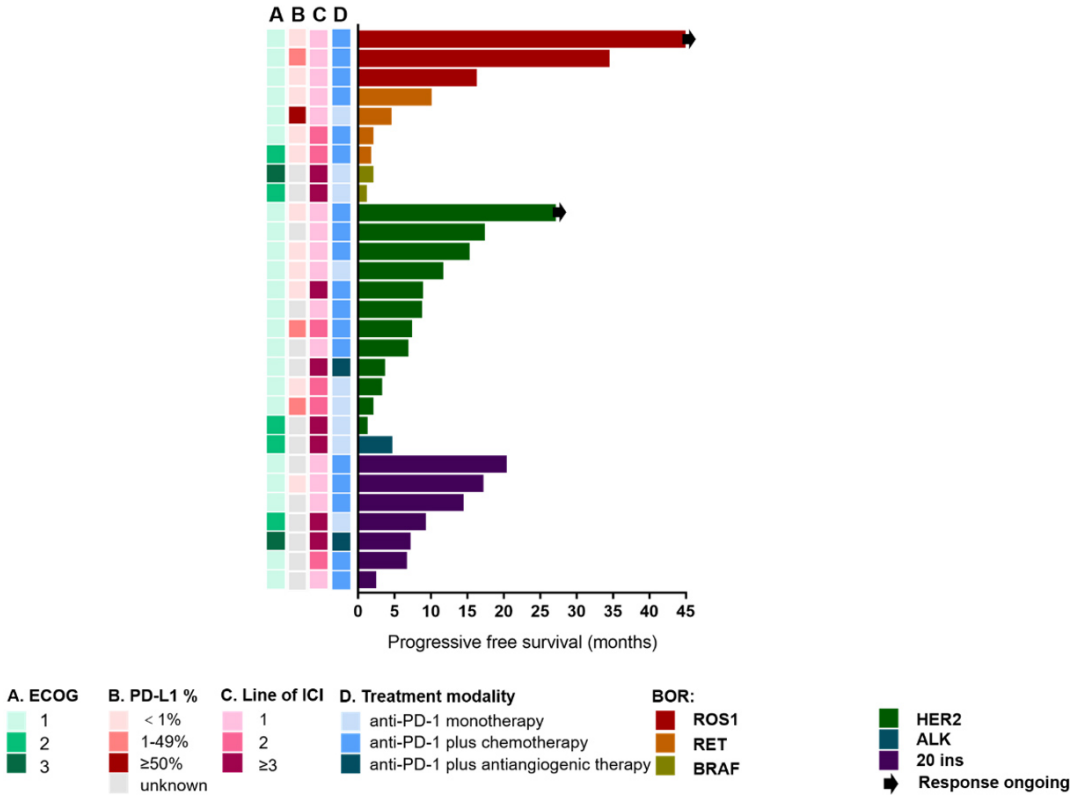

Figure 6. Swimmer's plot of PFS across molecular alterations. PFS: Progression-free survival; ALK: anaplastic lymphoma kinase; HER2: human epidermal growth factor receptor 2; BRAF: V-raf murine sarcoma viral oncogene homolog; RET: rearranged during transfection; ROS1: c-ros oncogene 1; ECOG: Eastern Cooperative Oncology Group; ICI: immune checkpoint blockade.

oncogene mutation on TME, studies have shown that EGFR TKIs, chemotherapy, and antiangiogenic therapy also influence the TME. EGFR TKIs could increase infiltration of CD8+ T cells, dendritic cells, and M1 TAMs and inhibit regulatory T cells infiltration[19]. Bevacizumab, an antiangiogenic therapy, has been observed in melanoma to increase circulating CD8+ T cells and interleukin-6 levels so as to change the TME in combination with chemotherapy[20]. Therefore, the use of ICI-based combined therapy as post-line treatment after TKI resistance may be more effective. In the IMpower150 study, we did see prolonged PFS and OS in the ABCP (atezolizumab, bevacizumab, carboplatin, and paclitaxel) group compared with the BCP (bevacizumab, carboplatin, and paclitaxel) group in patients with EGFR-driven NSCLC[21]. In our study, we also found ICI combined therapy was correlated with better outcome than ICI monotherapy in EGFR-driven NSCLC patients.

In our study, we did not find any correlation between KRAS mutation and better survival, which is inconsistent with previous studies. This may be explained by the heterogeneity of KRAS-mutant NSCLC. KRAS mutation subtypes include G12C, G12D, G12V, G12A, and G13D, among others, and different KRAS mutations can activate distinct signaling pathways, leading to different downstream effects, which may result in different response to therapies[22]. Besides, KRAS mutation is always accompanied by different patterns of co-occurring mutations, which display different immune profiles and show varying sensitivities to ICIs. In KRAS-TP53 co-mutated tumors, increased expression of PD-L1, higher TMB, and a remarkable clinical benefit of ICIs was observed[23,24]. Conversely, KRAS-STK11 has always been associated with poor clinical response to ICIs[24,25]. Further studies are needed to differentiate patients suitable for different treatment options.

HER2 mutations have been reported in approximately 2%-5% of lung adenocarcinomas[26,27] and correlated with poor prognosis[27]. The efficacy of ICIs in HER2-mutant NSCLC is ambiguous. Guisier *et al.*[28] reported 23 patients harboring HER2 mutation treated with ICI monotherapy who had a response rate of 27%, which is close to that observed in unselected patients with NSCLC. Mazieres *et al.*[13] reported an ORR of 7% and a median PFS of 2.5 months in 29 patients with HER2 mutation NSCLC treated with ICI monotherapy. In our study, ICI-based therapy displayed certain curative effect with 16.67% ORR, 91.7% DCR, and PFS of 7.8 months (range 1-26.9 months). For other rare driver mutations, the efficacy of ICIs on patients with ROS1 rearrangements or EGFR Exon20 insertion was rarely reported. In our study, three patients with ROS1 rearrangements had response of PR and durable PFS of 16.0, 34.2, and 45.0 months. Seven patients with EGFR Exon20 insertion had PFS of 9 months (range 2.2-20.1 months), ORR was 28.6%, and DCR was 85.7%. Therefore, ICI-based therapy may provide choices for these patients. As to BRAF, RET, and ALK, which represent a small subgroup of NSCLC, because of the limited number of patients, we could not draw a conclusion.

ICI-based combined therapy can bring benefit to patients with EGFR-mutant NSCLC. ICIs, especially ICI-based combination therapy, should not be excluded for patients with ROS1 rearrangement, HER2 mutation, and EGFR Exon20 insertion NSCLC. To achieve maximum benefit for these patients, better predictive biomarkers to select patients and combination modes of therapies should be explored.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Wu F, Zhang H, Guo X

Performed data acquisition, as well as provided administrative, technical, and material support: Du H, Yang M, Li J, Xiong A

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

The studies involving human participants were reviewed and approved by The Committee on Medical Ethics of Shanghai Pulmonary Hospital and written informed consent was obtained from all patients.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Perspective

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Epigenetic modulation in sensitizing metastatic sarcomas to therapies and overcoming resistance

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Abstract

Sarcomas are a class of rare malignancies of mesenchymal origin with a heterogeneous histological spectrum. They are classically associated with poor outcomes, especially once metastasized. A path to improving clinical outcomes may be made through modifying the epigenome, where a variety of sarcomas demonstrate changes that contribute to their oncogenic phenotypes. This Perspective article identifies and describes changes in the sarcoma genome, while discussing specific epigenetic changes and their effect on clinical outcomes. Clinical attempts at modulating epigenetics in sarcoma are reviewed, as well as potential implications of these studies. Epigenetic targets to reverse and delay chemotherapy resistance are discussed. Future directions with primary next steps are proposed to invigorate the current understanding of epigenetic biomarkers to enact targeted therapies to epigenetic phenotypes of sarcoma subtypes. Modifications to prior studies, as well as proposed clinical steps, are also addressed.

Keywords: Epigenetics, sarcoma, resistance, metastasis

INTRODUCTION

Transitioning cancer from a lethal disease to a chronic one necessitates long-term limiting of cancer growth. Underpinning mechanisms of growth and proliferation are classically thought of as activation of an

oncogene or the inhibition of a former tumor suppressor[1]. Some well-known examples of oncogenic driver mutations include EGFR-activation in non-small cell lung cancer and HER2 receptor amplification in breast cancer[2,3]. Pharmacological advances have led to the ability to target some of these drivers of tumor growth, resulting in significant clinical benefits. The development of imatinib is perhaps one of the best examples of this effect. After targeting the pathophysiologic driver BCR-ABL in chronic myeloid leukemia, complete response improved from 14.5% to 76.2%[4,5]. Imatinib is used in the management of gastrointestinal stromal tumors to target KIT or PDGFRα gain of function mutations which is present in the majority of tumors[6]. Imatinib has been reported to have a transformative effect on the median overall survival in patients with metastatic disease from months to years[7,8]. The possibility exists that some oncogene targets may be "undruggable" according to experts in the field, naturally leading to where the next best step may lie[9]. Mechanisms of resistance often develop to druggable targets via changes in drug metabolism, cell repair, and epigenetic effects[10]. Immunotherapy is emerging as a potential treatment option; however, response varies significantly depending on histological subtype and individual tumor pathology[11,12].

Sarcomas are a family of malignancies mostly derived from cells of mesenchymal origin[13]. Composed of more than 50 different subtypes, they are differentiated between soft tissue *vs.* bone origin, and up to 50% of patients develop metastatic disease, where median survival is 10-15 months[14]. Treatment of sarcomas remains difficult as the variety of histological subtypes demonstrates varying sensitivities to chemotherapies[15]. If localized, surgical resection and perioperative radiation remain the hallmark of treatment, whereas anthracycline-based chemotherapy predominates treatment of advanced soft tissue sarcoma[15-17]. Like soft tissue sarcoma, management of localized osteosarcomas also relies heavily on wide local resection of the tumor with a preoperative and postoperative multi-drug chemotherapy regimen (high-dose methotrexate, doxorubicin, and cisplatin)[18,19]. Treatment algorithms to both osteosarcoma and soft tissue sarcoma have remained the same over the past several years, with minimal improvements driving a desire for innovation[20,21].

Epigenetic modulation is a process linked to disease progression and therapeutic resistance (breast cancer, multiple myeloma)[22]. Dawson and Kouzarides[23] describe epigenetics as "chromatin-based events that regulate DNA-templated processes". Common mediators of epigenetics include histone de/acetylation and de/methylation, DNA de/methylation, and non-coding miRNAs that control mRNA translation without changing DNA cellular structure[24]. Cellular disruption of epigenetic control could be detrimental to long-term or induction treatment efficacy and is a postulated mechanism of resistance for various cancers[25,26]. One such example occurs in estrogen receptor-positive breast cancer, where the developed loss of *ARID1A* expression reduces the ability of estrogen to bind and thus decreases the therapeutic efficacy of selective estrogen receptor modulators[27]. Similar resistance effects have been reported when hypermethylation of DNA decreased the efficacy of cisplatin therapy in non-small cell lung cancer[28].

EPIGENETIC BACKGROUND OF SARCOMA

Soft tissue sarcomas

Epigenetic changes have been implicated in the proliferation of multiple soft tissue sarcomas (STS) subtypes through a variety of different epigenetic mechanisms[29]. Common contributors to STS progression include TP53 (47%), CDKN2A (22%), retinoblastoma 1 (RB1; 22%), neurofibromin 1 (NF1; 11%), and ATP-dependent helicase (ATRX; 11%)[30-32]. While mutational profiles of these genes have been characterized, recent efforts have improved our understanding of epigenetic alterations of these genes and the correlation to STS progression and prognosis[29-33]. For example, mechanisms of CDKN2A inactivation include histone post-translation modifications by polycomb repressive complex 2 (PRC2), hypermethylation of DNA at promoter CpG sites, and loss of function (LOF) mutations[32,34,35]. There have been several studies, including

a phase 2 clinical trial in liposarcoma and dedifferentiated liposarcoma, investigating the efficacy of cyclin-dependent kinase 4/6 inhibitors such as palbociclib in the setting of CDKN2A loss[36,37]. While the majority of STS with decreased CDKN2A expression exhibit LOF mutations in CDKN2A, loss of methylation in the CDKN2A promoter region has been shown to coincide with epigenetic transdifferentiation of uterine myosarcoma cells[38]. These findings suggest that hypomethylation in the CDKN2A promoter correlates with increased heterogeneity with hypomethylated tumors containing liposarcomatous and/or rhabdomyosarcomatous components[38]. Further investigation of DNA methylation in myosarcoma and other sarcomas could highlight novel mechanisms of tumor heterogeneity which have proven to be challenging to manage. Inhibition of other cell cycle players and their epigenetic modulators, such as the PRC2 and G2-checkpoint kinase WEE1, has also shown some preclinical efficacy in rhabdomyosarcoma (RMS)[39-41]. A recent publication by Zoroddu *et al.*[42] discussed the essential role of PRC2 in muscle cell differentiation, a key component of RMS progression, and a potential therapeutic avenue for RMS patients. Furthermore, targeting the catalytic subunit of PRC2, enhancer of zeste homolog 1/2 (EZH1/2), should be further explored in combination with inhibitors that target the cell cycle kinases CDK4/6 and Wee1.

Targeting epigenetic modulators at the DNA and histone levels has been reported to show efficacy in altering specific pathways implicated in STS pathogenesis as well as sensitizing sarcomas to radiation and chemotherapies. For example, synovial sarcoma (SS) is characterized by a chromosomal translocation t(X; 18)(p11.2; q11.2) that frequently results in an SS18-SSX fusion protein[43]. This fusion includes a subunit of BRG1/BRM associated factor, ATP-dependent chromatin remodel complex, and SS-associated t(X; 18) genes, respectively. This fusion has also been reported to alter epigenetic histone modulators [SWItch/Sucrose Non-Fermentable (SWI/SNF) complex, Polycomb Repressive Complex 1 (PRC1), and Polycomb Repressive Complex 2 (PRC2) complexes], ultimately resulting in hypermethylation and subsequent gene silencing[43]. Additionally, other sarcomas characterized by chromosomal translocations exhibit a similar effect on PRC2 and SWI/SNF, as mentioned, as well as histone deacetylases (HDAC)[44]. HDAC activity has been implicated in sarcoma pathogenesis and is associated with advanced disease and poor clinical outcomes[45]. For example, HDAC activity is shown to play a role in immunoreactivity (HDAC 1, 4, 6-8) in endometrial stromal sarcoma[45]. The use of HDAC inhibitors is a promising therapeutic option. HDAC inhibition has been shown to inhibit growth while sensitizing tumors to chemotherapy[45]. While a better understanding of chromosomal translocation-dependent epigenetic rewiring is needed, treatment of SS tumors could benefit from the use of epigenetic therapeutic agents like EZH1/2 and HDAC inhibitors.

Bone sarcomas

Investigation of epigenetics in bone sarcoma pathogenesis and potential treatment modalities has mainly focused on epigenetic mechanisms such as DNA methylation and histone modifications independently; however, mechanistic crosstalk between DNA methylation and histone modifications has been further elucidated in osteosarcomas and chondrosarcomas. A study by Li *et al.*[46] observed that treating osteosarcoma cells with 5-aza-2'-deoxycytidine (5-Aza-CdR, decitabine) enhanced radiosensitivity by inducing a G2/M checkpoint arrest and induced apoptosis. These studies alluded to 5-Aza-CdR induction of DNA damage sensitizing the osteosarcoma cells to radiation. Furthermore, a common epigenetic marker for DNA damage is γH2AX, a double-stranded break DNA damage-induced histone modification[47]. Future studies characterizing γH2AX as a biomarker for new treatment modalities such as DNA methylation inhibitors in combination with radiation therapy could be beneficial for stratifying which radiation-resistant tumors could be re-sensitized using this approach.

Chondrosarcoma (CS) is another bone sarcoma subtype for which DNA and histone methylation correlate and represent multiple angles to treating tumor resistance and recurrence. Mutations in isocitrate dehydrogenase, an enzyme involved in α-ketoglutarate (α-KG) production in the Krebs cycle, have been

documented in 52%-59% of central and 57% of dedifferentiated CS[48,49]. These mutations result in the production of an oncometabolite, δ-2-hydroxyglutarate, which is linked to a hypermethylated profile in CS tumors at the DNA/histone level, as well as promotion of malignant transformation[48]. Inhibiting both DNA methylation and histone methylation might be a useful approach to inhibiting the downstream function of this oncometabolite, known epigenetic compensatory mechanisms, and malignant transformation[48]. This concept has moved forward to clinical studies, where one such trial is evaluating the combination of HDAC inhibitor (belinostat) in combination with the hypomethylating agent guadecitabine (NCT04340843).

Ewing sarcoma is associated with a fusion oncoprotein Ewing Sarcoma breakpoint region 1-Friend leukemia integration 1 transcription factor (EWS-FLI1) that has been shown to be associated with epigenetic modulation, specifically DNA methylation, as well as histone acetylation and deacetylation[49,50]. Subsequent inhibition with sodium butyrate was also shown to be synergistic with vincristine, etoposide, and doxorubicin when grown *in vitro*[51]. Targeted inhibition of histone deacetylase likewise leads to a decrease in *in vitro* growth, reinforcing the potential promise of epigenetics in this specific cancer subtype[52].

DNA methylation-based classification of sarcoma

Sarcomas are a heterogeneous group stratified into many subtypes according to WHO criteria, which include distinct morphologies, immunohistochemistry, and disease-defining molecular events[53]. However, many subtypes lack unequivocal molecular hallmarks, resulting in discrepancies between diagnosis and treatment[53-56]. To help decrease these discrepancies, epigenetic changes such as DNA methylation could represent a resource to better distinguish sarcoma subtypes[39,57]. One study by Koelsche *et al.*[33] explored global DNA methylation levels using a machine learning algorithm to compare the methylome classification to initial diagnosis and using a publicly-available sarcoma classifier. Prototypical soft tissue and bone tumors were analyzed using either HumanMethylation450K BeadChip or the EPIC array. Following DNA methylome analysis of 1077 tumor cases, representing 54 histological types, 62 methylation classes were identified following unsupervised hierarchical clustering and t-Distributed Stochastic Neighbor Embedding. Identified methylation classes were defined as methylation corresponding to WHO classification (48/62), methylation corresponding with a subgroup of a WHO entity (9/62), methylation corresponding with two distinct WHO entities (3/62), or methylation representing a novel entity (2/62). The first novel classification included SARC (RMS-like), which exhibited rhabdomyoblast-like cell morphology and DICER1 mutations. The second novel classification included SARC (MPNST-like), which presents similarly to MPNST but retains H3K27me3, an epigenetic modification that alters DNA packaging and is lost in the majority of MPNST. This study identified distinct subgroups using DNA methylation that correlates with another epigenetic mechanism, histone methylation (H3K27me3). MPNST biology is one example in which DNA methylation and histone modifications have been correlated; however, little is known about the mechanisms of epigenetic crosstalk in sarcoma pathogenesis[58]. Paraffin-embedded and formalin-fixed tissues can be analyzed via HumanMethylation450K BeadChip or the EPIC array. Raw data is then uploaded and analyzed using the sarcoma classifier. This publicly available resource and ongoing research in epigenetic mechanisms of STS pathogenesis could greatly benefit STS patients as new therapeutic options become available. Understanding epigenetic mutations and mechanisms as well as emerging/available clinical applications could enhance STS standard of care, including diagnosis, prognosis, and treatment efficacy.

Additional preclinical considerations

While baseline epigenetic changes of STS and bone sarcoma have been more extensively studied, few preclinical models have been assessed with respect to sarcoma cells previously treated with standard-of-care regimens. However, this approach has shown promise in preclinical models of other cancers[59].

One study of melanoma demonstrates how combining epigenetic modulators with chemotherapy could be exploited in sarcoma with regard to acquired resistance[60]. Zakharia *et al.*[60] used a combination of the BRAF inhibitor vemurafenib with low-dose decitabine, a DNA methyltransferase inhibitor, in metastatic melanoma patients who had progressed on prior vemurafenib monotherapy. A fixed-dose of vemurafenib was combined with dose-escalated decitabine in a phase Ib trial design. Of the 14 patients enrolled in the trial, 3 achieved complete response, 3 achieved partial response, and 3 maintained stable disease. Clinical benefit rate was 79%, with no dose-limiting toxicities. Given the dramatic clinical responses seen in a refractory patient population, further preclinical studies were initiated to explore the effect. Decitabine was first confirmed to deplete DNA methyltransferase1 *in vitro* at concentrations below that needed to induce apoptotic effects. Separately, vemurafenib alone was able to inhibit cell growth until day 32, whereas the combined decitabine/vemurafenib regimen *in vitro* was able to maintain a cytostatic effect until day 90. Interestingly, cells in the combination treatment arm still demonstrated resistance to vemurafenib despite the prolonged stasis of cell growth in the combination treatment arm. Further analysis of patient genomics could shed light on whether non-responding patients had any shared features, though the structure of the study did not lend itself to outcome-driven analysis.

Previously performed clinical trials indicate that epigenetic modifiers within STS and bone sarcoma may demonstrate a similar effect. However, a critical area for growth is the creation of preclinical models that mimic treatment-experienced sarcoma models. Patients exposed to multiple lines of therapy in clinical settings do not have a corresponding or at least similarly derived cell line off of which treatments can be attempted. One could expect that epigenetic mechanisms similar to those seen in epithelial malignancies will have a better opportunity to be discovered and later reversed with the advent of better preclinical models[27,28].

PREVIOUS CLINICAL ATTEMPTS AT EPIGENETIC MODULATION

Soft tissue sarcomas

Epigenetic modulators as monotherapy for sarcoma have been attempted with some success, a selected summary of which is included in Table 1. Perhaps the best known is that of tazemetostat, an EZH2 inhibitor approved in unresectable epithelioid sarcoma, the first FDA-approved epigenetic therapy in a solid tumor[61]. Chu *et al.*[44] examined a pan-histone deacetylase inhibitor as monotherapy in 17 recurrent and metastatic soft tissue sarcoma patients. The best response in this study was stable disease in 8 patients, with no objective response seen. While limited in patient number, these two studies would suggest that unless the epigenetic target plays a role as an oncogenic driver, as in epithelioid sarcoma and EZH2 gain of function mutation, future efforts should focus on combination therapies with known cytotoxic agents as opposed to epigenetic targets as monotherapies[62].

Dembla *et al.*[63] enrolled 44 total patients, 26 diagnosed with STS, in a study evaluating the effect of pazopanib combined with either a histone deacetylase or inhibitors of mTOR, Her2, or MEK. The study was designed as a retrospective review of patients with metastatic sarcoma previously treated with, ideally, anthracycline-based chemotherapy analogous to the PALETTE study[64]. Half of the patients received histone deacetylase inhibitors. Overall survival and progression-free survival of STS patients taking pazopanib with an epigenetic modifier were 38.7 weeks and 14.5 weeks, respectively. Genomic analysis via the Cancer Genome Atlas was performed as well, demonstrating mutations in 46% of the targeted epigenomic modifiers (HDAC, mTOR, Her2, MEK). Significant co-alterations were discovered between the Ras/Raf/MAPK pathway and HDAC alterations as well as between the ERBB pathway and HDAC alterations, though the specific alterations were not reported, and STS were not differentiated from bone sarcomas. Conclusions of this study are difficult to draw, in part due to incompatibility with the reference

Table 1. A summary of the selected pharmaceutical trials highlighted by the epigenetic target and most prevalent sarcoma subtypes treated in the trial

Pharmaceutical(s)	Epigenetic target(s)	Sarcoma subtype(s)
Tazemetostat	EZH2	Epithelioid sarcoma
SB939	Histone deacetylase	Synovial sarcoma Myxoid liposarcoma Endometrial stromal Ewing sarcoma
Vorinostat	Histone deacetylase	Not specified
Valproate	Histone deacetylase	Uterine leiomyosarcoma Liposarcoma Angiosarcoma Epithelioid sarcoma
Decitabine	DNA methyltransferase	Leiomyosarcoma Chondrosarcoma Adenosarcoma Carcinosarcoma
Hydralazine Valproate	DNA methyltransferase Histone deacetylase	Not specified

standard overall survival of the PALETTE study, even after selecting for STS as bone sarcoma. Given that some patients in this study had undergone prior VEGF treatment, acquired resistance could explain this effect. The genomic analysis lends an opportunity to evaluate whether a synergistic effect exists between addressing multiple epigenetic pathways at once, especially the MAPK pathway and HDAC, as they were co-altered at a 97% rate. If the drop in reference to overall survival and this effect were mediated via epigenetics, it would also imply that reversing the resistance could be difficult (provided this resistance seen was due to the attempted mechanisms). As such, combining epigenetic modulators at the advent of treatment could be beneficial.

We, the authors, evaluated 46 patients diagnosed with unresectable metastatic STS with a combination regimen of gemcitabine/docetaxel, combination chemotherapy, VEGF inhibition with bevacizumab, and valproate as an HDAC inhibitor[65]. Nearly 74% of patients had received prior chemotherapy; 30% of patients had received prior gemcitabine and docetaxel. Seventeen patients required dose reduction from the study regimen, with main adverse events being hepatotoxicity, neurotoxicity, and hypertension. The best response within the trial was a complete response in a case of epithelioid sarcoma, as well as 6 partial responses in other sarcoma subtypes. Interestingly, of patients who had previously received combination gemcitabine and docetaxel, 61% responded to the addition of valproate and bevacizumab regimen with either partial response or stable disease. Bevacizumab as monotherapy has a 13% reported response rate in STS[66], and prior studies in treatment naïve metastatic STS patients demonstrated a 31% response rate to gemcitabine, docetaxel, and bevacizumab triple therapy[67]. With a response rate of 15%, of which 74% had previously undergone prior therapy, the rate of response in this trial is likely attributable to prior bevacizumab monotherapy in a majority of patients, reversal of chemotherapeutic resistance with bevacizumab, valproate or both, or effective valproate monotherapy.

Another phase 1b trial conducted by our group treated metastatic soft tissue and bone sarcoma with standard gemcitabine chemotherapy and the DNA hypomethylating agent decitabine[68]. Thirty-one patients (25 with STS) were enrolled, with 7 deemed non-evaluable; notably, 11 had received prior gemcitabine therapy with documented progression. Partial response was seen in 4 patients, with stable disease seen in 10, with a clinical benefit rate of 58%. Sarcoma subtypes with partial responses were leiomyosarcoma, adenosarcoma, and carcinosarcoma. These results combined with prior treatment with gemcitabine would imply that decitabine therapy was able to correct prior resistance to therapy.

An interesting, multifaceted approach in the setting of solid tumor treatment was performed by Bauman *et al.*[69]. From the perspective that aberrant DNA methylation is found in prior smokers, DNA methylation was used as a target in previously treated solid tumor patients. A combination regimen of hydralazine, demonstrated to be a demethylating agent *in vitro*, as well as valproate, demonstrated to have histone deacetylation activity, was utilized prior to chemotherapy in a steady state[70,71]. Patients were monitored for dose-limiting toxicities, and the regimen was well-tolerated, with no grade 4 side effects reported, and 5 total grade 3 side effects among 27 patients. Notable responses were seen in 2 of 3 STS patients who maintained stable disease for 3 and 4 months on second-line treatment. Further survival data of sarcoma patients was not reported. While the STS sample size was small, the data suggest a therapeutic benefit with combination epigenetic therapy that would be well-tolerated. Chavez-Blanco *et al.*[72] discovered a similar potentiation effect with xenograft fibrosarcoma cells in a murine model, revealing a higher antineoplastic effect of chemotherapy regardless of the mechanism if co-administered with hydralazine and valproate. Further, xenograft tumors undergoing combination therapy did not regrow after the final dose of treatment, whereas tumors treated with adriamycin monotherapy did recur, reinforcing a possible increased efficacy of the coadministration regimen.

Bone sarcomas

Chu *et al.*[44] used the same pan-histone deacetylase inhibitor monotherapy within 5 EWS-translocation sarcomas, 1 of which was diagnosed as Ewing's sarcoma, and 2 were diagnosed as chondrosarcoma. The patient in the study with Ewing's sarcoma had progression of disease whereas best response with chondrosarcoma had stable disease; EWS-translocation sarcomas had the highest percent tumor change from baseline compared to all other sarcoma subtypes in the study[44].

Dembla *et al.*[63] examined the effect of pazopanib combined with primarily histone de-acetylators or inhibitors of mTOR, Her2, or MEK. Overall survival and progression-free survival of osteosarcoma patients taking pazopanib with an epigenetic modifier were 35.2 weeks and 8.9 weeks, respectively. While referencing the PALETTE study in design, the PALETTE study did not include bone sarcomas in its original analysis. This cohort does not match expected pazopanib monotherapy overall survival and progression-free survival[73]. While possibly this is due to a detrimental effect of combination therapy with epigenetic modifiers, a genomic analysis via the Cancer Genome Atlas divided by sarcoma subtype beginning with soft tissue sarcoma and bone sarcoma could demonstrate a difference between the two groups[63].

Ongoing and recruiting clinical trials

Of the trials listed in Table 2, one important step forward involves the use of epigenetic modulators and immunotherapy. This is being used with EZH2 inhibitors, PARP-inhibitors, and DNMT inhibitors, with room for expansion into other epigenetic targets, especially if the results prove to be promising.

FUTURE DIRECTIONS

While a substantive amount of information relating to the role of epigenetics in the oncogenesis of sarcoma has only recently been discovered, further exploration to best derive benefit is still needed. Specifically, determining the epigenetic profile of an individual patient specimen is needed. While this is already being pursued in part by NCT03919539 in the evaluation of osteosarcoma, we suggest similar clinical studies in STS to better correlate epigenetic profile with disease progression, treatment susceptibilities with the current standard of care, and ultimately the development of targeted epigenetic therapies. Epigenetic profiling has already been shown to lead to the improved diagnostic characterization of sarcoma[33].

Table 2. Ongoing clinical trials evaluating epigenetics in the setting of sarcoma as documented by clinicaltrials.gov with associated clinical trial numbers

Clinical trial number	Tumor type(s)	Regimen	Epigenetic target	Study start date	Estimated end date
NCT04648826	Sarcoma, melanoma, germ cell	Aerosolized azacytidine and bintrafusp alfa	DNA methyltransferase	Oct 2021	Dec 2029
NCT04705818	Sarcoma, colorectal cancer, pancreatic cancer	Tazematostat and durvalumab	EZH2	Jul 2021	Jul 2023
NCT03694262	Endometrial sarcoma	Bevacizumab, atezolizumab and rucaparib	PARP-inhibitor	Jul 2019	Jun 2026
NCT03919539	Osteosarcoma	Famitinib and camrelizumab	Biomarker evaluation	Dec 2019	Dec 2021
NCT04340843	Chondrosarcoma	Belinostat, guadecitabine	DNA methyltransferase, histone deacetylase	Jul 2020	Jan 2022

Some positive outcomes in prior studies suggest epigenetic therapies can delay resistance to already effective chemotherapies, as demonstrated by Zakharia *et al.*[60] in the context of vemurafenib and melanoma, with preclinical correlates. To better elucidate these effects in sarcoma, we suggest a trial of patients with metastatic STS undergoing standard of care chemotherapy enrolled to receive concurrent epigenetic therapy to better potentially identify a prolonged duration of response. Currently, it also appears that epigenetic therapies are able to play a role in returning the effectiveness of a previously used regimen[63,65].

None of the epigenetic therapies used are able to specifically target only epigenomic changes related to sarcoma pathogenesis, leading to a possible concern over lack of specificity. Fortunately, one consistent determination among the studies reported here is that combination epigenetic therapy inhibiting multiple targets has thus far been well-tolerated by patients. We suggest combining current standard of care regimens with multiple epigenetic therapies as opposed to the monotherapies more frequently attempted, as these will inhibit any compensatory epigenetic changes seen in monotherapies. This will likely be more beneficial in the event further identification of the epigenome is performed and concurrent mutations are identified, as shown by Dembla *et al.*[63].

An additional approach, as seen in the NCT04648826 and NCT04705818 trials involving epigenetic targets and immunotherapy, would involve utilizing therapeutic options previously considered less effective with epigenetic targets to evaluate a change in efficacy. If effective, we hypothesize that the epigenetic target would likely unmask a therapeutic benefit similar to the re-sensitizing of chemotherapy, as shown by Monga *et al.*[65], though if the therapeutic benefit is found, more mechanistic characterization would need to be determined. One potential modification would be to utilize combination epigenetic targets as mentioned above.

The preclinical setting, as alluded to above, also has exciting potential for growth. Arguably one of the most beneficial changes would be the development of reflective treatment-experienced preclinical cell lines for STS and bone sarcomas for both characterization of suspected epigenetic mutations seen after treatment as well as reversing acquired resistance. Finally, many targets identified in preclinical settings have yet to be truly evaluated in a clinical context to determine their efficacy, as shown by Ciarapica *et al.*[40] who determined that inhibiting WEE1 was a promising effective therapy in high-risk RMS. This oncogenic pathway is shared by the PRC2 complex, leading to the possibility that a similar therapeutic effect may be found if the EZH2 inhibitor tazemetostat is used.

Multiple directions of study will lead to ample opportunity for growth in the study of epigenetics, as well as possibly defining the role that epigenetic modulation may play in the treatment of sarcoma.

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Authors' contributions

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Cancer Drug Resistance

Review

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Emerging actionable targets to treat therapy-resistant colorectal cancers

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Abstract

In the last two decades major improvements have been reached in the early diagnosis of colorectal cancer (CRC) and, besides chemotherapy, an ampler choice of therapeutic approaches is now available, including targeted and immunotherapy. Despite that, CRC remains a "big killer" mainly due to the development of resistance to therapies, especially when the disease is diagnosed after it is already metastatic. At the same time, our knowledge of the mechanisms underlying resistance has been rapidly expanding which allows the development of novel therapeutic options in order to overcome it. As far as resistance to chemotherapy is concerned, several contributors have been identified such as: intake/efflux systems upregulation; alterations in the DNA damage response, due to defect in the DNA checkpoint and repair systems; dysregulation of the expression of apoptotic/anti-apoptotic members of the BCL2 family; overexpression of oncogenic kinases; the presence of cancer stem cells; and the composition of the tumoral microenvironment and that of the gut microbiota. Interestingly, several mechanisms are also involved in the resistance to targeted and/or immunotherapy. For example, overexpression and/or hyperactivation and/or amplification of oncogenic kinases can sustain resistance to targeted therapy whereas the composition of the gut microbiota, as well as that of the tumoral niche, and defects in DNA repair systems are crucial for determining the response to immunotherapy. In this review we will make an overview of the main resistance mechanisms identified so far and of the new therapeutic approaches to overcome it.

Keywords: Colorectal cancer, resistance, chemotherapy, target therapy, EGFR, ERBB2, MET, BRAF, kinase inhibitors, immune checkpoint inhibitors, gut microbiota

INTRODUCTION

Notwithstanding widespread, effective measures of preventive screening, early detection and major advances in treatment options, colorectal cancer (CRC) is still the fourth cause of cancer-related death worldwide[1,2]. In fact, due to the relatively asymptomatic progression of the disease in the early stages, patients are frequently diagnosed with metastatic CRC (mCRC), with a 5-year survival rate of around 14%[3]. Neo-adjuvant or adjuvant chemotherapy, together with surgery, represent the backbone of the therapeutic approach for both early-stage and metastatic cancer patients. Depending on tumor site and progression of the disease, chemotherapy can be associated with radiotherapy[4-6] and, in selected cases, it can be possibly integrated with targeted therapy and/or immunotherapy. However, despite a variety of therapeutic approaches, resistance - both intrinsic and acquired - to drug treatment(s) remains one of the greatest challenges in the long-term management of incurable mCRC and eventually contributes to death[7,8]. In the last two decades, remarkable progresses have been made in the understanding of the mechanisms underlying drug resistance and in the unraveling of the feedback mechanisms fueling acquired resistance to targeted and/or immunotherapy. In addition, several phenotypes and synthetic lethality screens uncovered important liabilities that can be pharmacologically targeted in resistant tumors and in specific mutational settings[9]. Accordingly, hundreds of clinical trials are currently undergoing to test new drugs and new combinations for the treatment of mCRC. This review will pinpoint the most promising targets emerged in the last decade.

RESISTANCE TO CHEMOTHERAPY

According to the American Society of Clinical Oncology (ASCO) guidelines (https://www.asco.org/), the main drugs used for chemotherapy are 5-fluorouracil (5-FU), irinotecan, and oxaliplatin, either alone or in combinations such as in the FOLFOX (5-FU + leucovorin + oxaliplatin) or FOLFIRI (5-FU + leucovorin + irinotecan) regimens. 5-FU-based chemotherapy is the main approach for advanced CRC and, when used in combination, initial responses are up to 55%-65%. When administered sequentially, median overall survival (OS) can range from 18 to 20 months[10]. Although 5-FU initially de-bulks the tumor mass, recurrence usually occurs, thus pinpointing how acquired drug resistance, and consequent tumor re-growth, represents the main obstacle to effective clinical outcomes for CRC patients. Several different mechanisms have been identified accounting for resistance to 5-FU-based chemotherapy[11] and increasing number of strategies are being explored to increase the responsiveness of resistant tumors to chemotherapy [Figure 1].

Dysregulated expression of drug transporters and enzymes involved in drug metabolism

Plasma membrane transporters are pivotal for the uptake into and the efflux out of the cells of endogenous and exogenous molecules. They are divided in two big families: the ATP-binding cassette (ABC) family and the solute carrier (SLC) family. Members of both families serve a range of physiological roles but some of them also are determinants of drug disposition via affecting absorption, distribution, and excretion of drugs [Table 1].

Since the mid-1980s several members of the ABC family have been deemed to be play an important role in the resistance to chemotherapy, given that their dysregulated expression may lead to a decreased uptake or an increased efflux of anticancer agents[12-14]. Even though roughly half of the members have been shown to efflux anticancer agents in some context, the use of cell lines with high ABC transporter expression levels might have led to an overestimation of their role in cancer[15]. In addition, transporters are usually parts of more complex networks usually under the same transcriptional regulation; therefore, their upregulation may be more of an epi-phenomenon than of a cause-related effect[16]. A paradigmatic example of this are the data reported by Gao *et al.*[17]. They first showed that, in colon cancer cell lines made resistant to 5-FU, drug treatment induced the expression of different ABC transporters as well as the activation of IRE1, an enzyme

Table 1. Dysregulated expression of drug transporters and enzymes involved in drug metabolism

Gene	Protein function	Correlation with drug resistance	Ref.
SLC22A3	Uptake of platinum compounds	Lower SLC22A3 expression correlates with worst PSF in patients receiving FOLFOX6 regimen	[20]
		Post-treatment intracellular concentration of OxPt is higher in SLC22A3-overexpressing cells; upregulation of SLC22A3 in mouse xenografts rendered tumors more responsive to OxPt treatment	[21]
SLC31A1	Copper influx transporter, involved also in OxPt intake	SLC31A1 level predicts prolonged survival and enhanced response to platinum-based regimens in cancer patients with several epithelial cancers	[24]
ATP7B	Copper efflux transporter, involved also in OxPt efflux	Increased levels of ATP7B are associated with poor outcome in CRC patients receiving oxaliplatin-based chemotherapy	[23]
CYP1A2/ CYP2A6	Cytochrome P450 enzymes involved in drug metabolism	Increased expression in 5-FU-resistant HCT116 CRC cell line; addition of the CYP450 inhibitor phenylpyrrole enhanced 5-FU-induced cytotoxicity in 5-FU-resistant cells	[25]
CYP3A5		Higher intratumoral expression of CYP3A5 in patients with CRC who do not respond to irinotecan-based chemotherapy	[29]
TYMS	Enzyme that maintains the dTMP pool critical for DNA replication and repair and is inhibited by 5-FU	Increased expression of TYMS in pretreatment tumor biopsies identified tumors non-responsive to 5-FU-based therapy	[26]

PSF: Progression-free survival; OxPt: oxaliplatin; 5-FU: 5-fluorouracil; FOLFOX: 5-FU + leucovorin + oxaliplatin.

involved in the unfolded protein response ensuing from endoplasmic reticulum stress[18]. Disabling the enzyme, by both a specific inhibitor and by RNA silencing, they observed a decrease in the expression of the transporters as well as the sensitization to 5-FU. Given that the unfolded protein response is a complex response where several genes are regulated downstream of IRE1[19], their experiments do not rule out that the sensitization might be due to the suppression (or activation) of other components of the response. Therefore, the decrease in the expression of the transporters simply correlates with the sensitization effect without a clear cause-effect relationship having been established.

For decades now plenty of experimental data have been produced - both in *in vitro* and *in vivo* systems - showing that the increased expression of several members correlate with increased resistance to chemotherapeutic agents as well as their inhibition by specific inhibitors could restore drug sensitivity[12,13]. Although the substrates and key roles for most of these transporters have been identified, the extent to which these transporters play an effective role in clinical multidrug resistance has not yet been clarified[15,16], not in general nor in the specific case of CRC. In fact, *ex-vivo* data are still pretty inconclusive: for example ABCB1 expression is generally low in tumors, but for few exceptions, and specific inhibitors have clinically failed[16].

On the other hand, some members of the SLC family 22 shown to be involved in the uptake of platinum compounds have been more directly linked to drug efficacy. For example, in a retrospective study Gu *et al.*[20] found that high expression of SLC22A3 (OCT3) may be a protective factor for CRC patients postoperatively treated with FOLFOX6 as a first-line adjuvant chemotherapy. In line with this, the same group also demonstrated in *in vitro* and *in vivo* experimental systems that the cellular concentration of oxaliplatin and its cytotoxicity were significantly increased in response to high expression of OCT3, whereas OCT3 knockdown directly increased the invasion and migration of colon cancer cells. In addition, upregulation of OCT3 expression in colon cancer xenografts via treatment with the DNA methyltransferase inhibitor decitabine increased the cellular concentration of the drug and improved its curative effect[21]. Key mediators for oxaliplatin accumulation inside the cells are also a series of proteins initially identified as copper transporters. It has been shown that the major copper influx transporter SLC31A1 (CTR1) regulates tumor cell uptake whereas the two copper efflux transporters ATP7A and ATP7B regulate the efflux[22].

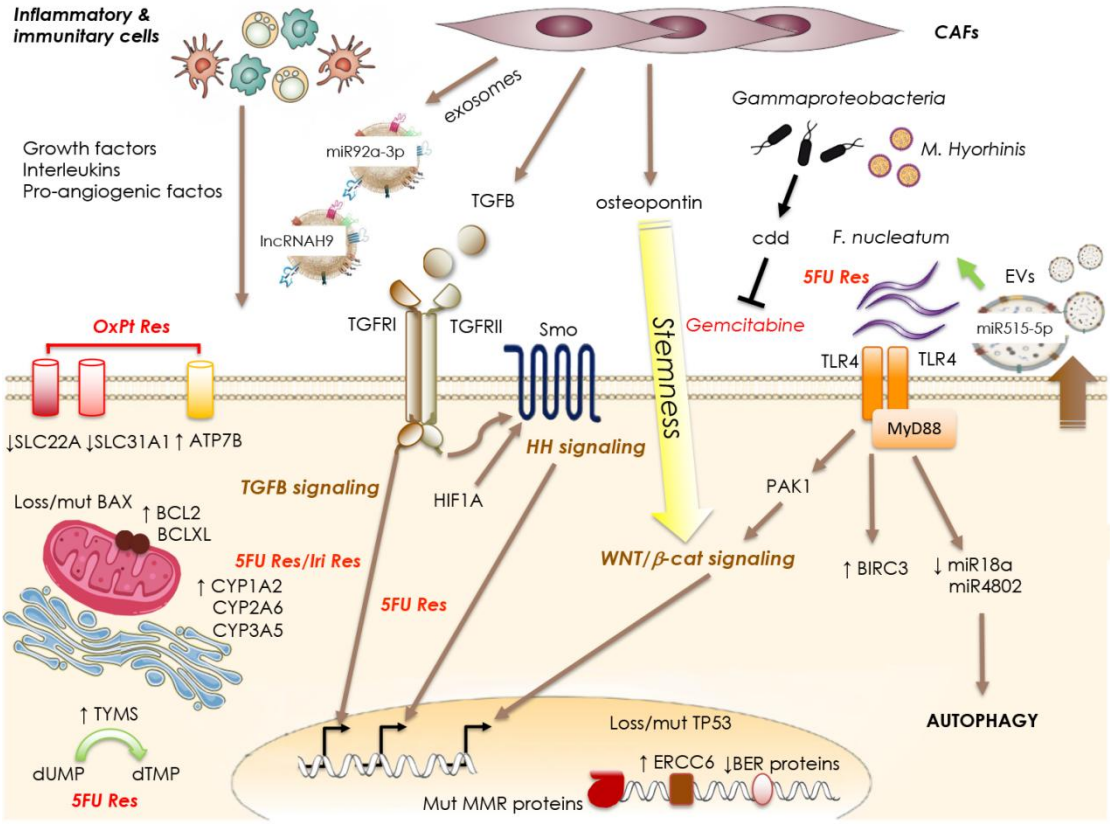

Figure 1. Mechanisms of resistance to chemotherapy. Tumor cell-intrinsic mechanisms may involve: dysregulated expression of drug transporters (SLC family members or ATP7B) and enzymes involved in drug metabolism (CYP family members), imbalanced expression of anti-/pro-apoptotic molecules (BAX mutation/loss or increased BCL2 expression), or dysregulation of DNA repair mechanisms and checkpoints (*TP53* mutation/loss, MMR proteins mutation, diminished expression of BER proteins, or increased ERCC6 expression). External signals acting on the tumor cells to trigger resistance may derive from the cells populating the tumoral niche such as the CAFs releasing TGFB, osteopontin and exosomes containing specific lncRNAs and miRs. In addition, inflammatory and immunitary cells of the niche release a number of interleukins, growth factors, pro-angiogenic factors. Also, specific components of the microbiota can contribute to the resistance to chemotherapy by directly inactivating the drug (Gammaproteobacterial or *M. hyorhinis*) or by engaging the TLR4/MyD88 system to transduce pro-survival and autophagic signals. SLC: Solute carrier; MMR: mismatch-repair system; CAFs: cancer-associated fibroblast.

Accordingly, it has been reported that increased levels of ATP7B are associated with poor outcome in CRC patients receiving oxaliplatin-based chemotherapy[23], whereas a large meta-analysis of literatures and datasets performed by Sun *et al.*[24] revealed that high CTR1 level predicts prolonged survival and enhanced response to platinum-based regimens in cancer patients with a number of epithelial cancers.

A change in the expression or activation of enzymes involved in drug metabolism by cancer cells can enhance the catabolism of the drugs or result in diminished activation of prodrugs. In both cases, the result is an impairment of the pharmacological action of the chemotherapeutic agent [Table 1]. For example, Untereiner *et al.*[25] produced a 5-FU-resistant HCT116 CRC cell line characterized by a significant increase in the expression of the drug-metabolizing cytochrome P450 enzymes CYP1A2 and CYP2A6. Accordingly, they reported that the addition of the CYP450 inhibitor phenylpyrrole enhanced 5-FU-induced cytotoxicity in 5-FU-resistant cells[25]. Also, increased expression of thymidylate synthase (the enzyme inhibited by 5-FU) in pretreatment tumor biopsies could identify tumors that would be non-responsive to 5-FU-based therapy[26]. At variance, downregulation of thymidine phosphorylase (which plays an essential role in activating the oral prodrug capecitabine to 5-FU) causes resistance via insufficient drug activation[27].

Irinotecan undergoes extensive metabolism in both the liver and the intestine; it is converted to inactive metabolites by CYP3A4 and CYP3A5[28]. Studying a CRC patient cohort, Buck *et al.*[29] recently reported an association between the response to irinotecan and the expression CYP3A5; in fact, they found a significantly higher intratumoral expression of CYP3A5 in patients with CRC who do not respond to irinotecan-based chemotherapy and thus suggested a causal role of CYP3A5 in tumor resistance.

Dysregulation of DNA repair mechanisms and checkpoints

5-FU and all the most used drugs for treating CRC act by inducing, directly or indirectly, DNA damage and consequently activate cell's DNA damage response. Depending on the entity of the damage and on the functionality of the DNA damage response, apoptosis can be the outcome. In fact, several DNA repair mechanisms can intervene to remove or repair drug-induced damage thus avoiding cytotoxicity, and their dysregulation can contribute to the drug-resistant phenotype [Table 2]. For example, ERCC6 - a member of the excision repair cross-complementation (ERCC) family of enzymes, involved in the nucleotide excision repair pathway (NER) - is upregulated in CRC tissues compared to matched non-tumoral adjacent tissues and is also upregulated in patients resistant to 5-FU treatment. Conversely, low ERCC6 expression is associated with better response to chemotherapy and survival in CRC[30].

Activity of the base-excision repair (BER) system is pivotal in determining 5-FU-induced cytotoxicity in cells concomitantly defective for components of the mismatch-repair system (dMMR), as shown by the fact that CRC patients expressing high levels of BER proteins have more aggressive tumors and poor outcomes after chemotherapy[31]. Of note, zelpolib, a specific inhibitor of DNA polymerase δ an essential component of both NER and BER pathways - has been recently synthesized and shown to render cells sensitive to PARP inhibitors[32]. Therefore, it would be of interest to assess whether PARP inhibitors are cytotoxic in ERCC6-overexpressing and MMR/BER defective 5-FU-resistant models.

MMR status is particularly important in CRC given that around 15% of patients have one or more components (*MLH1*, *MLH3*, *MSH2*, or *MSH3*) mutated or silenced, often with a microsatellite instability (MSI) phenotype. Notably, patients with stages II and III CRC are less responsive, if not at all, to 5-FU-based chemotherapy whereas a better response is achievable in patients with MMR-proficient (pMMR) tumors[33,34]. In addition, MMR phenotype is also predictive of resistance to oxaliplatin-based chemotherapy[35]. Notably, collections of evidence are accumulating that dMMR and MSI-high (MSI-H) heavily pre-treated patients (at least two prior lines of therapy for metastatic disease) show a durable clinical benefit when treated with programmed cell death protein 1 (PD-1) inhibitors, particularly in the metastatic setting[36].

The response to DNA damage is tightly controlled by DNA damage checkpoints and their misfunctioning may allow the survival of damaged cells and the selection of new mutations. In this case, the resistance to chemotherapy as well as the tumor progression are favored characteristics. The best-known DNA damage checkpoint, mutated or lost in 50% to 70% of CRC cases, is *TP53* which makes it the gene with the highest mutation rate in CRC[37,38]. Loss or impairment of *TP53* have been shown to affect the response to chemotherapy in several *in vitro* and *in vivo* systems[37], to predict the poor response of patients with mCRC treated with chemotherapy[38], and be associated with poor survival after FOLFOX therapy[39,40].

Many promising synthetic lethal vulnerabilities, whose targeting kills p53-null drug resistant-resistant CRC cells, have been identified by an shRNA screen performed in our lab[41]. Several of them have successively been validated by other labs such as PIM-1[42], TRIB3[43], EPHA2[44,45], CHK1[46], and VEGFR2[47]. We focused our studies particularly on two targets: GSK3B and p65BTK. We showed that GSK3B is significantly more

Table 2. Dysregulation of DNA repair mechanisms and checkpoints

Gene/system	Protein function	Correlation with drug resistance	Ref.
ERCC6	Member of ERCC family of enzymes involved in the NER	Upregulated in CRC tissues compared to matched non-tumoral adjacent tissues Higher levels in patients resistant to 5-FU treatment low expression associated with better response to chemotherapy and survival	[30]
BER	Members of the BER system repairs bulky helix-distorting lesions	CRC patients with defects in components of the mismatch-repair system and expressing high levels of BER proteins have more aggressive tumors and poor outcomes after chemotherapy	[31]
MMR	Members of MMR are involved in recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases	dMMR patients with stages II and III CRC are less responsive, if not at all, to 5-FU-based chemotherapy whereas a better response is achievable in pMMR patients	[33,34]
TP53	DNA damage checkpoint	Loss or impairment of TP53 affects the response to chemotherapy in several *in vitro* and *in vivo* systems	[37]
		Loss or impairment of TP53 predict the poor response of patients with mCRC treated with chemotherapy	[38]
		Loss or impairment of TP53 associated with poor survival after FOLFOX therapyLoss or impairment of TP53 associated with poor survival after FOLFOX therapy	[39,40]

ERCC: Excision repair cross-complementation; NER: nucleotide excision repair pathway; BER: base-excision repair; MMR: mismatch-repair system; dMMR: defective MMR; pMMR: proficient MMR.

activated in drug-resistant *vs.* responsive CRC patients and is associated with cancer progression, poor response to therapy, and worse OS. Experimentally, we demonstrated that in the absence of p53, GSK3B activity allows cells to survive, despite treatment with DNA-damaging drugs, by sustaining DNA repair and that its downregulation restored sensitivity to 5-FU of p53-null colon cancer cells - both in *in vitro* and in *in vivo* models - via induction of regulated necrosis[48]. Moreover, we also demonstrated that GSK3A is redundant with GSK3B modulating drug resistance and chemotherapy-induced regulated necrosis[49]. A general role for GSK3B in sustaining resistance to chemotherapy has been validated also in other types of cancers and 9-ING-41, a novel GSK3B inhibitor developed by actuate therapeutics, is currently undergoing phase 1 and phase 2 trials, as a single agent and in combination with cytotoxic agents, in patients with refractory cancers, including CRCs[9]. p65BTK is a novel isoform of the Bruton's tyrosine kinase we isolated from the screening and subsequently characterized. Compared to the known Bruton's tyrosine kinase (BTK) expressed in bone marrow-derived cell lineages, p65BTK lacks a stretch of 86 amino acids at the N-term and is translated from an mRNA containing a different first exon. Protein abundance is regulated at the post-transcriptional level and under the control of RAS/ERK pathway. Moreover, p65BTK is endowed with strong transforming activity that depends on ERK1/2 and its inhibition abolishes RAS transforming activity. Accordingly, p65BTK overexpression in CRC tissues correlates with ERK1/2 activation[50], histotype, and cancer progression[51]. p65BTK silencing or chemical inhibition affects the growth of CRC cells and overcomes the 5-FU resistance of p53-null CRC cells by abolishing a 5-FU-elicited TGFB1 protective response and triggering E2F-dependent apoptosis. In addition, the combination of BTK inhibitors with 5-FU is cytotoxic for p53-null patient-derived organoids and significantly reduced the growth of xenografted tumors, thus giving the proof-of-concept for suggesting the use of BTK inhibitors in combination with 5-FU as a novel therapeutic approach in CRC patients[51]. Further studies from our lab indicate p65BTK as an actionable target in different solid tumors other than CRC. In fact, preclinical data indicate that depending on the tumor type, BTK inhibitors used alone can induce cytotoxicity in gliomas[52], be more effective than standard-of-care (SOC) chemotherapy in drug-resistant ovarian cancer[53] or can kill drug-resistant tumor cells when used in combination with SOC chemotherapy or targeted therapy in non-small-cell lung cancer[54], and melanomas (unpublished data).

Interestingly, its targeting is effective in cells with p53 loss and defects along the RAS/MAPK pathway, making it an actionable target for a broad range of solid tumors resistant to SOC chemotherapy. Notably several drugs targeting BTK are already in clinic for certain types of B-cell malignancies (e.g., ibrutinib, acalabrutinib, and zanubrutinib), whereas several others are in clinical trial for B-cell malignancies (e.g., orelabrutinib, spebrutinib, pirtobrutinib, and BGB-3111) and for different type of diseases characterized by excessive B-cell activation (e.g., remibrutinib, renebrutinib, tolebrutinib, and rilzabrunib). Thus, assessing their efficacy in drug-resistant CRCs and other solid tumors would be feasible and advantageous.

Imbalanced expression of anti-/pro-apoptotic molecules

Drug resistance has been demonstrated to be associated with low levels of the pro-apoptotic BAX protein or its loss[55,56], which can occur in CRC patients because of inactivating mutation or somatic frameshift mutation in the dMMR setting[57]. In a low-expressing experimental CRC model, its re-induction by using andrographolide, a natural diterpenoid, restored the apoptotic response to 5-FU of drug-resistant CRC cells[58]. Drug resistance has also been associated with increased levels of the anti-apoptotic BCL-XL protein, which largely occurs in CRC either by amplification or by overexpression[59]. Several molecules antagonizing BCL-XL, the so-called BH3-mimetics, are currently being tested in experimental models in combination with chemo- or targeted therapy. For example, ABT-737 has been shown to improve the response to oxaliplatin in a *TP53*wt/*KRAS*mut background[60]. Both ABT-737 and WEHI-539 have been shown to lower the apoptotic threshold by increasing the mitochondrial priming, thus sensitizing resistant CRC cells to chemotherapy; also, Ch282-5 potentiated the response to oxaliplatin both *in vitro* and *in vivo*[61]. On the other hand, ABT-263 has been proven to overcome hypoxia-driven radioresistance and improve radiotherapy[62]. Several BH-3 mimetics are currently being tested in clinical trials, even though not for CRC, but mainly for hematological malignancies and other solid tumors[59]. Hence, it may be hypothesized that in a near future these molecules will also be tested in CRC patients.

Cancer stem cells and the tumoral niche

It has been repeatedly demonstrated that in the bulk of the tumor population, a very small fraction is represented by cancer stem cells (CSCs) that are intrinsically resistant to chemotherapy. In addition, upon exposure to anticancer agents and radiotherapy they may enter a quiescent state, resistant to anti-proliferative drugs; once the therapy is suspended, they can self-renew and differentiate into heterogeneous lineages of cancer cells, thus driving tumor recurrence[63]. Initially, several data indicated that CSCs upregulate drug-efflux pumps, have a superior DNA-repair capacity, and have enhanced antioxidant defenses[64]. More recently, cell plasticity and, in particular, the ability of CSCs to adopt a quiescent state have also emerged as important drivers of drug resistance. In fact, lineage-tracing approaches have revealed that the potential of committed cells to move up and down the hierarchy of differentiation ("plasticity") is more widespread than previously thought. Interestingly, several studies have provided evidence that both CSCs and non-CSCs are plastic and capable of undergoing phenotypic transitions in response to appropriate stimuli[64]. Therefore, under stimuli coming from the tumoral niche - composed of mesenchymal cells, tumor-infiltrating immune cells (TIIC), endothelial cells, extracellular matrix, and inflammatory mediators - the stemness phenotype can be acquired also by non-CSCs. In particular, cancer cells that can enter a reversible drug-tolerant "persister" state in response to treatment have been described[65]; this population is made of cycling and non-cycling persisters which arise from different cell lineages with distinct transcriptional and metabolic programs. Upregulation of antioxidant gene programs and a metabolic shift to fatty acid oxidation has been associated with persister proliferative capacity across multiple cancer types, including CRC. Notably, using different inhibitors to impede oxidative stress or metabolic reprogramming led to a significant reduction in the fraction of cycling persisters[66].

In CRC it has been shown that chemotherapy enriches for cells with a CSC phenotype. However, a pivotal role for a full definition of functional stem cell is concomitantly played by tumor microenvironment (TME) properties and in particular by osteopontin - a multifunctional protein that can also act as a cytokine - produced by key cancer-associated fibroblasts (CAFs)[67]. Recently, an escape mechanism leading to tumor re-growth after 5-FU treatment has been identified in a p53-mediated activation of the WNT/beta-catenin signaling, a pivotal pathway to sustain CRC CSCs. Accordingly, the addition of a WNT inhibitor to 5-FU effectively suppressed the CSCs and reduced tumor re-growth after discontinuing the treatment[68]. Also, CRC CSCs can escape immune surveillance by avoiding recognition by the innate immune system and shape the TME through the release of exosomes, cytokines, and chemokines to generate an immunosuppressive niche that facilitates cancer progression[69].

As mentioned above, TME cues from the tumoral niche can support drug resistance. Important players in this scenario are CAFs, which represent an essential component of tumoral stroma and produce several cytokines acting on tumor cells. Beyond osteopontin, another multifunctional cytokine released by CAFs is transforming growth factor beta (TGFB) that can act synergistically with hypoxia-induced tumor cell-expressed HIF1A to sustain 5-FU/oxaliplatin resistance via activation of the hedgehog pathway, as demonstrated by *in vitro* and *in vivo* experiments using patient-derived cells[70]. Interestingly, 5-FU-induced TGFB production occurs also in CRC cells as a mechanism of resistance and targeting TGFBRI restores the sensitivity of drug-resistant cells to 5-FU toxicity[71]. In addition, the 5-FU-elicited TGFB1 protective action can also be abolished by the use of BTK inhibitors[51]. Other than through cytokine release, CAFs can communicate with the cancer cell directly, by transferring exosomes. For example, it has been shown that exosomal transfer of miR-92a-3p to CRC cells activates the WNT/beta-catenin pathway and inhibits mitochondrial apoptosis, and contributes to cell stemness, epithelial-to-mesenchymal transition, metastasis, and resistance to 5-FU/oxaliplatin treatment[72]. Also, exosomal transfer of lncRNA H19 has been found to promote the stemness and chemo-resistance of CRC via activation of the WNT/beta-catenin pathway due to its action as a competing endogenous RNA sponge for miR-141[73]. Notably, these data further indicate WNT/beta-catenin pathway as a potentially actionable target to overcome chemotherapy resistance.

Exosomes can also be exchanged by cancer cells themselves either in normal conditions or upon chemotherapy. For example, it has been shown that miR-21 is significantly upregulated in exosomes purified from CRC cell lines and that adding them to the cells induces resistance against 5-FU through the downregulation of PDCD4[74]. Exosomes derived from 5-FU-resistant cells instead are enriched in growth/differentiation factor 15 (GDF15) and dipeptidyl peptidase IV (DPP4), both of which proved to be potent inducers of angiogenesis[75,76]. Accordingly, 5-FU-resistant CRC cells showed high microvascular density *in vivo*[75]. Taken together these data indicate that GDF15 and DPP4 may be novel targets for inhibiting angiogenesis in 5-FU-resistant colon cancers.

Finally, in the tumoral niche, different types of TIICs are present that interact with cancer cells and the other components of the niche through cytokine production, eventually altering tumor growth and its response to drug therapy[69,77]. TIICs in the TME have dual functions in cancer progression: TIIC-related inflammation facilitates tumorigenesis, but TIICs also harbor antitumor properties when appropriately activated. Cancer cell-secreted factors hijack TIIC functions to promote tumor development and metastasis and to suppress immune recognition[69]. Accordingly, one of the most important progress in cancer treatment has been the recent addition to chemotherapy of the so-called immune checkpoint (ICIs) inhibitors that act by interrupting the immunosuppressive signals within the TME and reactivating antitumor immunity[78].

The gut microbiota

Gut microbiota seems to be implicated in chemotherapy efficacy through numerous mechanisms [Table 3], including xenometabolism, immune interactions, and altered community structure. Evidence suggests a potential relationship between the presence of *Fusobacterium nucleatum* and resistance to 5-FU and oxaliplatin chemotherapy and no response to immunotherapy. For example, Yu *et al.*[79] reported that *F. nucleatum* was abundant in CRC tissues in patients with recurrence post-chemotherapy and associated with clinicopathological characteristics. Mechanistically, F. nucleatum targeted TLR4 and MYD88 innate immune signaling and specific microRNAs to activate the autophagy pathway and support drug resistance[79]. Other mechanisms by which *F. nucleatum* confers resistance to chemotherapy have been reported, such as the upregulation of BIRC3 expression (a member of the inhibitor of apoptosis family, IAPs)[80] and the activation of the WNT/beta-catenin pathway via a TLR4/P-PAK1 cascade[81]. In an animal model of CRC, the intratumor presence of bacteria belonging to the Gammaproteobacteria class and producing the long form of the bacterial enzyme cytidine deaminase, which mediates gemcitabine deamination, conferred resistance to gemcitabine; accordingly, the elimination of bacteria by ciprofloxacin treatment restored the response to chemotherapy. In addition, the presence of *Mycoplasma hyorhinis* determined resistance to gemcitabine[82]. At variance, responses to oxaliplatin were reduced in efficacy in tumor-bearing mice that lacked microbiota[83] indicating that the microbial population resident in the gut can affect both sensitivity and resistance to chemotherapy, being these effects modulated by different bacteria.

Finally, it has been recently shown that commensal bacteria can produce extracellular vesicles (EVs) able to deliver to the human cells bacterial RNA molecules with gene expression regulatory ability, thus suggesting that they might potentially regulate the expression of genes involved in drug resistance. The communication between commensal bacteria and host cells have been shown to be bi-directional suggesting a reciprocal regulation of gene expression and accordingly, biological cell function. An example of this communication having an impact in the development of drug resistance is offered by the previously mentioned study by Yu *et al.*[79], where resistance to 5-FU and oxaliplatin has been shown to be mediated by *F. nucleatus*-induced autophagy through the TLR4/MYD88-mediated downregulation of miR-18a* and miR-4802. Conversely also intestinal epithelial cells exerted a reciprocal effect onto *F. nucleatus* by controlling its growth through the delivery of specific miRNAs, such as hsa-miR-515-5p. In CRC patients, altered expression of miR-515-5p might thus affect *F. nucleatus* proliferation and consequent response to chemotherapeutic drugs[79]. EV-mediated intercellular communication between bacteria and cancer cells seems therefore to be another potential mechanism playing a role in determining the efficacy of cancer therapy and worthy of increasing attention.

RESISTANCE TO TARGETED THERAPY

According to ASCO guidelines, chemotherapy can be possibly integrated with anti-angiogenic agents such as bevacizumab or aflibercept (first-line and second-line treatment, for advanced CRC) and regorafenib (in patients with mCRC who have already received chemotherapy and other targeted therapies). In selected cases, chemotherapy may also be integrated with the addition of targeted therapy. For example, anti-EGFR (anti-epidermal growth factor receptor) monoclonal antibodies (e.g., cetuximab and panitumumab) are indicated only for *RAS*- and *BRAF*-wild type (wt) tumors since the mutation of these two genes confer resistance to the EGFR blockade. In addition, a combination using the BRAF inhibitor encorafenib and cetuximab may be used to treat patients with *BRAF*-mutant (mut) mCRC who have received at least one previous treatment. In the last decade, several tumor-expressed "immune checkpoints", immunosuppressive molecules blocking antitumor immunity, have been identified and "checkpoint inhibitors" have been developed and entered the clinic. Among them are antibodies against programmed cell PD-1 or its ligand PD-L1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). So far, immunotherapy has a limited use

Table 3. The gut microbiota and the response to chemotherapy/immunotherapy

Bacterium	Correlation with drug resistance	Mechanism	Ref.
Fusobacterium nucleatum	Abundant in CRC tissues of patients with recurrence post-chemotherapy and associated with clinicopathological characteristics	Targeting of TLR4 and MYD88 innate immune signaling and targeting specific microRNAs to activate the autophagy pathway	[79]
		Upregulation of BIRC3 expression	[80]
		Activation of the WNT/beta-catenin pathway via a TLR4/P-PAK1 cascade	[81]
Gammaproteobacteria ***Mycoplasma hyorhinis***	Resistance to gemcitabine reverted by eliminating bacteria by ciprofloxacin treatment	Production of the long form of the bacterial enzyme cytidine deaminase which mediates gemcitabine deamination	[82]
Whole microbiota	Reduction of the response to oxaliplatin in mice lacking microbiota		[83]
Bacteroides thetaiotaomicron Bacteroides fragilis	Their introduction in SPF mice models of CRC restored the response to anti-CTLA-4 treatment, absent in germ-free or antibiotic treated mice		[147]

SPF: Specific pathogen-free.

in CRC since PD-1 targeting monoclonal antibodies can be used only in selected cases of mCRCs bearing defects along the DNA damage response pathways. For example, pembrolizumab is used for the therapy of mCRCs that are MSI-H or dMMR. In contrast, nivolumab can be administered to patients with dMMR or MSI-H mCRC that has grown or spread after treatment with chemotherapy, either alone or in combination with ipilimumab (anti-CTLA-4 monoclonal antibody). Notably, the FDA has issued its approval for pembrolizumab as the first-line treatment of patients with dMMR/MSI-H mCRC in June 2020, even though neither the Japanese Pharmaceuticals and Medical Devices Agency nor the European Medicines Agency has yet approved pembrolizumab as the frontline regimen[84].

To expand the range of possible combinations of precision drugs to add to chemotherapy several efforts have been made to better stratify patients and to understand the basis of resistance/insensitivity to targeted therapy.

Resistance to anti-EGFR blockade

Perhaps the best studied so far are the mechanisms and the determinants underlying the resistance to anti-EGFR drugs [Figure 2], given that they have been the first targeted drugs employed for the treatment of CRC, and are so far the most used [Tables 4 and 5]. The main genetic defects hampering the response to EGFR blockade are activating mutations leading to constitutive activation of signaling pathways downstream of EGFR such as mutations in *KRAS* (30%-40%), *NRAS* (4%), *BRAF* (7%-15%), or *PIK3CA* (17%-30%) genes and *PTEN* deletion or truncation (7%) which altogether account for unresponsiveness in around 70% of cases[85,86]. It has been reported that in approximately 50% of *RAS*-wt patients, sensitivity to EGFR blockade is lost because of secondary occurring mutations[87]. In a small fraction of the remaining resistant tumors two important biomarkers are emerging. Bardelli *et al.*[88] initially reported that certain patients harboring *RAS*-wt gene and resistant to anti-EGFR therapy presented an amplification of the *MET* proto-oncogene that proved to be responsible for *de novo* and acquired resistance to anti-EGFR therapy. In fact, functional studies showed that MET activation conferred resistance to anti-EGFR therapy both *in vitro* and *in vivo* and could be overcome using MET inhibitors[88]. Remarkably, examining circulating tumor DNA, it was possible to find MET amplification even before the recurrence became clinically evident. Much experimental evidence further supported the benefit of targeting MET in anti-EGFR-resistant CRCs and explored the mechanisms driven by MET. Among them, acquisition of cetuximab resistance was shown to be mediated by TGFA overexpression which in turn induced EGFR-MET interaction[89]. Also, SRC activation promoted cetuximab resistance by directly interacting with MET; accordingly, pretreatment with

Table 4. Resistance to anti-EGFR blockade

Treatment	Mutational background	Mechanism	Ref.
Cetuximab	Activating mutations in *KRAS*, *NRAS*, *BRAF* or *PIK3CA* genes and *PTEN* deletion, truncation, or epigenetic silencing	Constitutive activation of signaling pathways downstream of EGFR	[85,86]
Cetuximab Panitumumab	*MET* amplification in wt*KRAS* setting	Activation of signaling pathways converging onto PIK3CA and MAPK; resistance bypassed using MET inhibitors	[88]
Cetuximab		TGFA overexpression which in turn induced EGFR-MET interaction	[89]
Cetuximab		Cetuximab-mediated MET activation via interaction with SRC and abolished by SRC inhibitors	[90]
Cetuximab	*ERBB2* amplification /mutations in quadruple (*KRAS*, *NRAS*, *BRAF*, and *PIK3CA*) wt setting	Constitutive activation of signaling pathways downstream of the receptor; resistance overcome by addition of anti-ERBB2 agents	[97,98]
Cetuximab	*ERBB2* amplification and heregulin overexpression	ERBB2/ERBB3 dimerization; resistance bypassed by silencing ERBB2 and depleting heregulin	[101]
Intrinsic resistance to anti-EGFR agents	*ERBB2* activating mutations in *KRAS*-wt mCRCs	Constitutive activation of signaling pathways downstream of the receptor; resistance bypassed by dual targeting of ERBB2 via trastuzumab and lapatinib	[103]
Cetuximab+Refametinib	*KRAS*-mut	PI3K-AKT pathway activation ensuing from autocrine loops and heterodimerization of multiple receptors (ERBB2, ERBB3, and IGF1R)	[112]
Cetuximab+FOLFIRI	*RAS*-wt mCRC	High levels of EPHA2 significantly correlated with worse PSF and increased progression rate in a cohort of patients; primary and acquired resistance to cetuximab reverted in in *in vitro* and *in vivo* models by adding a specific EPHA2 inhibitor	[114]

FOLFIRI: Folinic acid + 5-fluorouracil + irinotecan; PSF: progression free survival.

SRC inhibitors abolished cetuximab-mediated MET activation and rendered CRC cells sensitive to cetuximab[90]. Interestingly, concurrent inhibition of MET and SRC decreased viability and enhanced apoptosis in both *RAS*-wt and *RAS*-mut CRC cells. Therefore, combined inhibition of MET and SRC may be a promising strategy for treating of CRC, independent of anti-EGFR resistance[91]. In addition, MET inhibition, by enhancing the formation of DNA double-strand breaks and possibly alleviating tumor hypoxia, has been found to sensitize CRC cells also to irradiation[92], further expanding the actionability of MET in CRCs.

Despite experimental efficacy, the clinical trials performed so far did not confirm the expectations. In a phase 2 study the addition of the specific MET inhibitor tivantinib to cetuximab allowed only 10% of MET-high, *KRAS*-wt mCRC patients to achieve objective response, even though in a difficult-to-treat setting (tumor progression on cetuximab or panitumumab after a first-line chemotherapy)[93].

In another phase 1/2 study addition of tivantinib to cetuximab + irinotecan was tested in *KRAS*-wt patients previously treated but who progressed or presented with mCRC. In these cases, the combination did not significantly improve progression-free survival (PFS), even though subgroup analyses trended in favor of tivantinib in patients with MET-high tumors, PTEN-low tumors, or those pretreated with oxaliplatin[94]. One possible explanation for these disappointing

Table 5. Strategies to overcome resistance to EGFR inhibition

Drug(s)	Mutational background	Mechanism	Ref.
Cetuximab + Crizotinib	*MET* amplification	Double targeting of EGFR and MET	[88]
Cetuximab + PHA665752	Overexpression of TGF-α	Double targeting of EGFR and MET	[89]
Cetuximab + PHA665752 Cetuximab + Dasatinib	*RAS*-wt; *MET* activation	Double targeting of EGFR and MET Double targeting of EGFR and SRC	[90]
Cetuximab + Lapatinib Cetuximab + Pertuzumab	*ERBB2* amplification	Double targeting of EGFR and ERBB2	[97]
Trastuzumab + Neratinib Trastuzumab + Afatinib	*ERBB2* amplification or mutation	Double targeting of ERBB2	[98]
Trastuzumab + Lapatinib (clinical trial)	*ERBB2* overexpression; *RAS*-wt	Double targeting of ERBB2	[103]
Cetuximab + ALW-II-41-27	EPHA2 overexpression	Double targeting of EGFR and EPHA2	[114]

PHA665752: Small-molecule inhibitor of MET; ALW-II-41-27: EPH family tyrosine kinase inhibitor.

clinical results might be that clinical efficacy has been obscured by a lack of standardization in MET assessment for patient stratification. Accordingly, a very recent paper indicated that subtyping of MET expression may be required to identify MET-addicted malignancies in CRC patients who will truly benefit from MET inhibition[95].

In 2011 *ERBB2* gene amplification was reported in 7% of patients with CRC[96]. Subsequently, *ERBB2* amplification or somatic mutations have been described by several studies, with highly different reported rates of positivity (up to 50%) likely due to several factors such as cohort heterogeneity, a small study population, antibody clone selection, staining platform, and different scoring system. Using a molecularly annotated platform of 85 mCRC patient-derived xenografts, Bertotti *et al.*[97] identified *ERBB2* amplification as an actionable liability in cetuximab-resistant CRCs harboring wt *KRAS*/*NRAS*/*BRAF*/*PIK3CA*. Moreover, they showed that the addition of anti-ERBB2 agents to anti-EGFR monoclonal antibodies overcame resistance and inhibited tumor growth[97]. The same group also showed that tumor growth was reduced by single targeting of ERBB2 in cetuximab-resistant, quadruple (i.e., *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*) wt CRC patient derived xenografts with *ERBB2* mutations. However, only double targeting of EGFR and ERBB2 led to durable tumor regression[98].

Later, the role of *ERBB2* amplification/mutation in predicting response to anti-EGFR treatment was confirmed by different groups. Cremolini *et al.*[99], conducted a prospective, case-control study to demonstrate the negative predictive impact of a panel of rare genomic alteration including *ERRB2* amplification/activating mutations, *MET* amplification, *ROS1*/*NTRK1-3*/*RET* rearrangements, and *PIK3CA* exon 20, *PTEN*, and *ALK* mutations. Of the resistant cases, 51.1% were associated with one of these candidate molecular alterations, *ERBB2* amplification being the most frequent (14.9%), followed by *MET* amplification (8.5%)[99]. A retrospective study performed in *RAS*/*BRAF*-wt mCRC patients reported that anti-EFGR therapy allowed a PFS significantly longer in patients without *ERBB2* amplification than in those bearing the alteration[100]. Shorter PFS and OS were demonstrated also in cetuximab-treated CRC patients with *ERBB2*-amplified tumors and higher serum heregulin levels. In addition, *in vitro* experiments proved that silencing ERBB2 overexpression, as well as depleting heregulin - thus disrupting of ERBB2/ERBB3 heterodimerization - restored the response to cetuximab[101]. Notably, ERBB2 amplification could be identified non only in tissues but also in circulating tumor DNA of CRC patients non-responsive to anti-EGFR therapy[102]. Sartore-Bianchi *et al.*[103], demonstrated that *ERBB2* activating mutations in *KRAS*-wt mCRCs predicted intrinsic resistance to anti-EGFR agents that could be bypassed by combining dual targeting ERBB2 via trastuzumab (anti-ERBB2 monoclonal antibody) and lapatinib (dual EGFR and ERBB2 tyrosine kinase inhibitor). A phase 2 basket trial recently obtained an overall response rate of 32% when

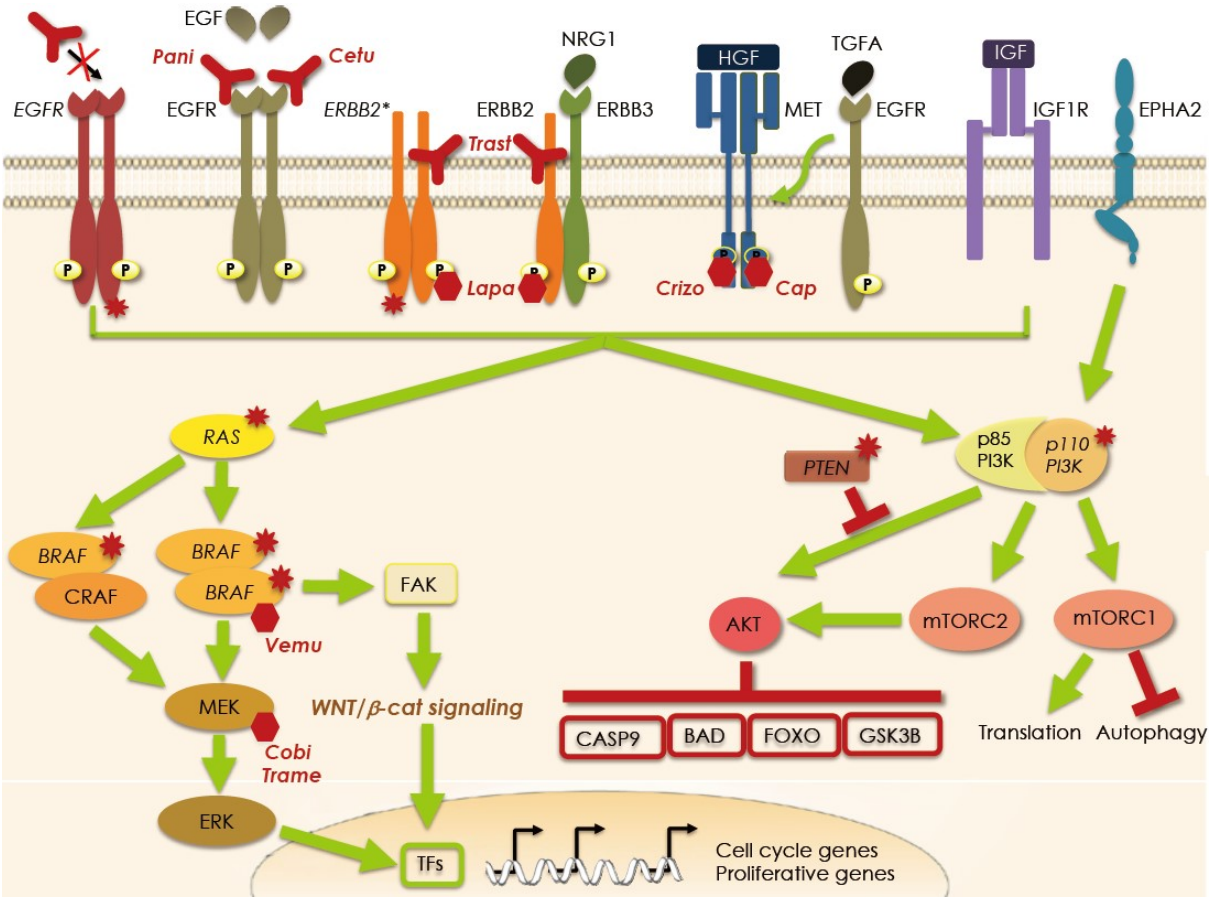

Figure 2. Mechanisms of resistance to targeted therapy against EGFR and mutated *BRAF*. Intrinsic resistance to EGFR blockade by panitumumab (Pani) and cetuximab (Cetu) occurs in case of activating mutations in the *EGFR* and its downstream effectors such as RAS, BRAF, and PIK3CA and when there is loss of *PTEN* either due to truncation or deletion. Acquired resistance stems from stimulation of collateral pathways impinging on the same downstream signaling activated by EGFR (i.e., the RAS/MAPK and the PIK3/AKT pathways). Hyperactivation of these pathways may be due to overexpression or mutation of *ERBB2*, heterodimerization of ERBB2 and ERBB3, engagement of the EGFR by TGFA which in turn activates MET, MET overexpression, EPHA2 overexpression, or IGF1R engagement. Strategies to overcome the insensitivity to EGFR blockade are directed at the inhibition of the collateral signaling such as using ERBB2 blocking agents trastuzumab (Trast) and lapatinib (Lapa) and the MET inhibitors crizotinib (Crizo) and capmatinib (Cap). Resistance to the mutated BRAF inhibitor vemurafenib (Vemu) originates from the shifting in the choice of the dimerization companion. In the resistant cells instead of mutated BRAF homodimers, mutated BRAF/CRAF heterodimers are formed which are insensitive to the inhibitor and thus MAPK activation is refueled. To prevent hetodimerization a novel inhibitor, that specifically bind mutated BRAF homodimers has been developed. Another route of escape to mutant BRAF blockade has been described where activation of the WNT/β-catenin signaling is triggered via FAK. Both MAPK and β-catenin signaling eventually activate a series of transcription factors (TFs) responsible for the transcription of proliferative genes. Currently, to prevent resistance to BRAF inhibition, a vertical blockade of the pathway is being tested in clinical trials using vemurafenib in combination with a MEK inhibitor such as cobimetinib (Cobi) or trametinib (Trame). Mutated proteins are indicated by the red stars and the italicized name.

using the dual targeting approach (pertuzumab and trastuzumab) in pretreated *ERBB2*-amplified mCRC patients[104]. In addition, two other phase 2 clinical trials also identified ERBB2 as a druggable target in refractory *KRAS*-wt mCRCs with *ERBB2* amplification[105,106], and ERBB2-targeted combinations have been recently included in international guidelines for mCRC[107].

Besides the above-mentioned clinical studies, several trials are currently undergoing where ERRB2-targeted antibody-drug conjugates (i.e., TDM-1, DS-8201, A166, ZW25, and ZW49) and novel tyrosine kinase inhibitors (i.e., tucatinib, sapitinib, neratinib, pyrotinib, poziotinib, and ceralasertib) are being tested alone

or in different combinations[108]. A recent study pinpointed that an optimal combination may require triple targeting. Mangiapane *et al.*[109], using 60 CRC specimens obtained a collection of CSC-derived spheres which was then used to perform preclinical experiments to validate different therapeutic options. They found that high expression levels of ERBB2 occurred in spheres showing hyperactive PI3K/AKT pathway and proved that triple targeting of ERBB2, MEK and PI3K induces CSC death and regression of anti-EGFR-resistant tumor xenografts, including those carrying *KRAS* and *PIK3CA* mutation[109].

Even though MAPK deregulation plays a pivotal role in both intrinsic and secondary resistance to anti-EGFR agents, other mechanisms (sometimes co-occurring) contribute or can account for secondary resistance, due to CRC molecular heterogeneity[110]. For example, stimulation of EGFR results not only in the RAS/RAF/MAPK pathway but also in the PTEN-PI3K-AKT cascade activation[111].

Recently, Vitiello *et al.*[112] used four different *KRAS*-mut CRC cell lines rendered resistant to a combination of cetuximab and refametinib (a selective MEK-inhibitor) and demonstrated that PI3K-AKT pathway activation acts as an escape mechanism in this setting. They showed that autocrine loops and heterodimerization of multiple receptors lead to the activation of several receptor tyrosine kinase, such as ERBB2, ERBB3 and IGF1R, whose signaling onto the PI3K/AKT pathway allows to bypass the block imposed by the double targeting of EGFR and MEK[112]. The effect on the response to cetuximab in the setting of the three most common *AKT1* activating mutations found in CRC patients (i.e., E17K, E49K, and L52R) was recently investigated by overexpressing them in a cetuximab-sensitive *KRAS*-wt CRC cell line. Interestingly, all of them impaired the cytotoxic response not only toward cetuximab but also to chemotherapy. In addition, also the most common mutations of *CTNNB1* (i.e., T41A, S45F, and S33P) were found to sustain resistance to the same agents[113]. These data indicate that possible therapeutic strategies to counteract the resistance to EGFR-targeted therapy would therefore be the use of inhibitors of the PI3K/AKT pathway. Notably, trials to test AKT and PI3K inhibitors for CRC are currently undergoing.

A novel predictive biomarker of resistance and a potential therapeutic target for improving the response to anti-EGFR agents has recently been identified in EPHA2. Studying a cohort of 82 *RAS*-wt mCRC patients treated with FOLFIRI + cetuximab, Martini *et al.*[114] found that 62.6% of patients expressed the tyrosine kinase receptor and that high levels of EPHA2 significantly correlated with worse PFS and increased progression rate. In *in vitro* models a specific EPHA2 inhibitor reverted primary and acquired resistance to cetuximab causing cell growth inhibition, inducing apoptosis and cell-cycle G1-G2 arrest. In xenografts experiments the treatment with the inhibitor upon progression with cetuximab significantly inhibited tumor growth[114].

Resistance to BRAF inhibitors

The BRAF kinase, operating downstream of RAS along the pathway leading to ERK activation, is considered an oncogenic driver in CRC and its mutation arises in 7%-15% of patients with mCRC. Over 95% of *BRAF* mutations in mCRC occur in codon 600 (V600E) whereas non-V600E *BRAF* mutations occur only in about 2% of patients and define a clinically distinct subtype with a better prognosis. In general, patients with *BRAF*-mut CRC have impaired survival not only in the metastatic setting but also in non-metastatic disease, as compared with patients with *BRAF*-wt CRC, and are resistant to chemotherapy. In fact, the median OS is 4 to 6 months after failure of initial therapy. Currently, SOC is an aggressive strategy involving triplet chemotherapy and anti-VEGF agents, but no specific tailored therapeutic approach has been standardized since no optimal combinations have been reported yet. In fact, at variance with what was observed for melanoma, vemurafenib - the first specific $BRAF^{V600E}$ inhibitor tested - showed modest clinical activity in mCRC patients with *BRAF*-mut tumors[115]. In addition, no responses were observed in the 10 patients with

BRAF^{V600E} mCRC enrolled in a phase 2 basket trial[116]. Overall, only 5% of patients with *BRAF*^{V600E} CRC respond to BRAF inhibitors[117] indicating a very high level of intrinsic resistance to its blockade [Tables 6 and 7]. The reason for the unresponsiveness to vemurafenib was uncovered in two key studies where a paradoxical effect ensuing from BRAF inhibition was shown in *BRAF*^{V600E} CRC models. BRAF^{V600E} can signal as RAS-independent monomers or dimers - depending on levels of RAS activation in the tumor - but predominantly exists as a drug-sensitive monomer. Vemurafenib selectively inhibits BRAF monomers. It was discovered that BRAF^{V600E} inhibition suppressed ERK-mediated negative feedback on EGFR activity, thus leading to EGFR-mediated reactivation of MAPK signaling, due to the formation of active RAF and CRAF protein dimers[118,119]. Recently, PLX8394, a novel experimental BRAF inhibitor has been reported to inhibit ERK signaling by specifically disrupting BRAF-containing dimers, thus acting on tumors driven by dimeric BRAF mutants and opening a new possibility to overcome resistance to classic BRAF inhibitors[120]. Interestingly, few possible targets for combinatorial treatment have been experimentally identified in *BRAF*^{V600E} CRCs. For example, it has been shown that in this setting anti-apoptotic MCL1 is upregulated due to ERK-mediated phosphorylation which results in stabilization of the protein. Even though MEK inhibitor cobimetinib suppressed MCL-1 phosphorylation to a greater extent than did vemurafenib, it resulted in only mildly cytotoxic effects. At variance co-targeting MEK and MCL-1 (either via silencing or via a small-molecule antagonist) significantly increased cytotoxicity *in vitro* and reduced tumor growth *in vivo*[121]. Chen *et al.*[122] demonstrated that in *BRAF*^{V600E} CRC cell lines BRAF inhibitors upregulated the WNT/beta-catenin pathway through activating FAK independently of EGFR and MEK signaling [Figure 2]. Accordingly, combined inhibition of BRAF/WNT pathways or BRAF/FAK pathways exerted strong synergistic antitumor both *in vitro* and *in vivo*[122]. Recently, another mechanism of resistance to vemurafenib has been identified in the increased abundance and activity of nucleophosmin (NPM1), a protein that regulate centrosome duplication and histone assembly and that is induced by the inhibitor in resistant cells. Accordingly, pharmacological inhibition of NPM1 effectively restored the sensitivity of vemurafenib-resistant *BRAF*-mut CRC cell lines[123].

Given the EGFR reactivation observed in BRAF inhibitor-treated CRC models, dual targeting has been tested. Adding cetuximab to vemurafenib in xenografts experiments resulted in increased antitumor activity and improved survival[124] and combined administration of BRAF and EGFR inhibitors induces tumor regression in most patients[116,125-127]. However, within 6 months, resistance inevitably occurs. Analyzing matched tumor tissues obtained from eight patients before treatment and at the time of disease progression after therapy, Yaeger *et al.*[128] found that in resistant tumors new alterations occurred in genes that encode components of the RAS/RAF pathway (activating mutations of *KRAS* or *NRAS* or amplification of *NRAS*-wt, *KRAS*-wt, or *BRAF*^{V600E}). They then overexpressed *NRAS*-wt or *KRAS* wt in *BRAF*^{V600E} CRC cell lines sensitive to vemurafenib/cetuximab treatment, demonstrating that the increase in either of the two was sufficient to induce the presence of RAF and CRAF protein dimers (absent in sensitive cells) thus rendering the cells resistant to the combinatorial therapy[128]. Therefore, it would be interesting to verify in this setting the effect of the inhibitor PLX8394 that acts by disrupting dimers[120].

Preclinical and clinical data obtained in *BRAF*^{V600E} melanoma models and patients indicated that simultaneous and vertical targeting of more than one node along the MAPK/ERK pathway, such as BRAF and MEK, could bypass the resistance to BRAF inhibitors[129,130] and delay the onset of relapse. However, also this combination did not live up to expectations in *BRAF*^{V600E} CRCs. In fact, combination treatment with BRAF plus MEK inhibitors, dabrafenib and trametinib, led to a median PFS of 3.5 months, with 56% of patients achieving stable disease, 9.3% partial response, and only 2.3% complete response[131]. It has been recently shown that the combination of the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib triggered a massive apoptotic response only in *BRAF*^{V600E} CRC cells harboring also *TERT* promoter

Table 6. Resistance to BRAF inhibitors

BRAFi	Mutational background	Mechanism	Ref.
Vemurafenib	$BRAF^{V600E}$	Only BRAF monomers inhibited by vemurafenib; EGFR-mediated reactivation of MAPK signaling, due to the formation of active RAF and CRAF protein dimers	[118, 119]
Vemurafenib	$BRAF^{V600E}$	Upregulation of Wnt/β-catenin pathway through activation of FAK	[122]
Vemurafenib	$BRAF^{V600E}$	Upregulation of NPM1	[123]

Table 7. Strategies to overcome resistance to BRAF inhibitors

Drug(s)	Mutational background	Mechanism	Ref.
PLX8394	$BRAF^{V600E}$	Specific inhibition of BRAF dimers; prevent EGFR-mediated reactivation of MAPK signaling	[120]
Cobimetinib + A-1210477	$BRAF^{V600E}$	Double targeting of MEK and MCL-1	[121]
Vemurafenib + PF-562271 Vemurafenib + ICG-001 Vemurafenib + PF-562271 + trametinib Vemurafenib + ICG-001 + trametinib	$BRAF^{V600E}$	Double targeting of $BRAF^{V600E}$ and FAK Double targeting of $BRAF^{V600E}$ and β-catenin Triple targeting of $BRAF^{V600E}$, FAK, and MEK Triple targeting of $BRAF^{V600E}$, β-cat, and MEK	[122]
Vemurafenib + NSC348884	$BRAF^{V600E}$	Double targeting of $BRAF^{V600E}$ and NPM1	[123]
Encorafenib + Cetuximab (clinical trial)	$BRAF^{V600E}$	Double targeting of $BRAF^{V600E}$ and EGFR	[136]

PLX8394: RAF inhibitor; A-1210477: elective MCL-1 inhibitor; PF-562271: FAK inhibitor; ICG-001: beta-catenin inhibitor; NSC348884: nucleophosmin (NPM1) inhibitor.

mutations but not in $BRAF^{V600E}$ cells lacking *TERT* mutations. Accordingly, the combination of inhibitors nearly completely abolished the growth of xenografted tumors $BRAF^{V600E}$/*TERT*-mut but had little effect on tumors harboring only $BRAF^{V600E}$. Notably, after the treatment was discontinued, doubly mutated tumors remained barely measurable whereas $BRAF^{V600E}$ tumors regrew rapidly[132]. These findings suggest that double testing for $BRAF^{V600E}$ and *TERT* mutations may stratify patients for combinatorial therapy with BRAF plus MEK inhibitors.

Recent clinical trials investigated the benefit of targeting different nodes along the MAPK pathway in *BRAF*-mut mCRC[133-135]. BEACON III was a three-arm phase 3 study that enrolled patients with $BRAF^{V600E}$ mCRC tumors who progressed on one or two prior regimens. In this setting the safety and efficacy of encorafenib (BRAF inhibitor) and cetuximab with or without binimetinib (MEK inhibitor) were evaluated and compared with the control arm with SOC therapy (irinotecan/cetuximab or FOLFIRI/cetuximab). Both triplet and doublet therapy induced an overall response rate that was 10 times more than in the control arm. In addition, PFS tripled and mean OS doubled for the doublet and triplet arms *vs.* control arm. Little difference was observed between doublet and triplet arms[136]. Based on these results, on April 2020, the FDA approved encorafenib in combination with cetuximab for mCRCs with a $BRAF^{V600E}$ mutation[137].

A final note, not directly related to resistance but pointing to alternative actionable targets in $BRAF^{V600E}$ CRCs, should be made about immunotherapy. As previously mentioned, therapy with ICIs is limited to mCRCs bearing defects along DNA damage response pathways. Notably, around 60% of *BRAF*-mut CRCs also are MSI-H, a condition associated with a higher proportion of tumor infiltrating lymphocytes present in the tumor[138]. Therefore, it is reasonable to suggest that ICIs could represent the new SOC for this subgroup[139].

Resistance to anti-angiogenic therapy

The first anti-angiogenic agent entering the clinic for the treatment of mCRC has been bevacizumab, a humanized monoclonal antibody anti-VEGF-A which binds circulating VEGF-A and blocks the interaction with its cell surface receptors (VEGFR1 and 2), thus reducing microvascular growth and inhibiting the blood supply to the tumor tissues[140]. In recent years the development of anti-angiogenic agents has increased, leading to the addition to of aflibercept (second-line treatment for advanced CRC) and regorafenib (in patients with mCRC who have already received chemotherapy and other targeted therapies). Aflibercept is a recombinant fusion protein consisting of VEGF-binding portions from the extracellular domains of human VEGFR1 and 2, that are fused to the Fc portion of the human IgG1 immunoglobulin and acts like a "VEGF trap". Regorafenib is multi-kinase inhibitor targeting angiogenic, stromal, and oncogenic receptor tyrosine kinase that shows strong anti-angiogenic activity due to its dual targeted VEGFR2-TIE2 tyrosine kinase inhibition. In addition, other anti-angiogenic drugs are in preclinical and clinical phase I-III studies[141].

Despite initial promising preclinical results, anti-angiogenic monotherapies did not lead to many clinical benefits, due to primary or acquired resistance, through activation of alternative mechanisms that support vascularization and tumor growth [Figure 3]. Clinical data show that anti-angiogenesis therapies can prolong PFS but have a limited impact on OS and do not set a permanent cure in CRC[142]. This limited clinical significance is likely due to the fact that treatment with antiangiogenic agents causes a discontinuity in the blood vessel network, which leads to a new hypoxic condition with activation of vascular mimicry, alternative pro-angiogenic pathways and recruitment of endothelial cells derived from bone marrow[143].

So far, the mechanisms of resistance to anti-VEGF therapy have not been fully elucidated. The main factor used by cancer cells to adapt to oxygen deprivation is hypoxia-inducible factor (HIF) of which three members are known (i.e., HIF1A, HIF2A, and HIF3A). During hypoxia, HIF1A translocates into the cell nucleus to form the activated HIF1 complex along with HIF1B. Hypoxic contexts induce upregulation of VEGF expression through the upstream transcription factor HIF1A. These factors induce tumors to acquire more angiogenic and invasive potentials, promoting metastasis[144]. Preclinical studies and clinical trials suggest that inhibition of a specific growth factor can induce the expression of others[145]. For instance, in a phase II study in which mCRC patients were treated with a combination of 5-FU, leucovorin and irinotecan (FOLFIRI) and bevacizumab, several angiogenic factors, including the placental growth factor and hepatocyte growth factor, were found to increase before disease progression[146]. Activin receptor-like kinase 1 (ACVRL1) is an emerging target for antiangiogenic therapy; it is a TGFB transmembrane receptor expressed preferentially from proliferating endothelial cells and is crucial to TGFB-mediated angiogenesis. Significantly, VEGF-A and ACVRL1 expression have been shown to regulate angiogenesis. Data support the hypothesis that ACVRL1 expression and function following anti-VEGF/VEGFR inhibitor treatment may contribute to vascular adaptation from the VEGF-driven neovascularization[147]. In particular, the TGFB axis may provide an escape pathway for tumor angiogenesis[148]. Thus, agents that inhibit TGFB signaling combined with VEGF/VEGFR- therapies may potentiate treatment response.

Moreover, alternative angiogenic pathway can contribute to the escape from anti-VEGF treatment such as the angiopoietin-TIE signaling system. TIE-1 and -2 are specific vascular receptor tyrosine kinase that control vascular permeability and the development and remodeling of blood vessels through angiopoietin-1 (ANGPT1) and angiopoietin-2 (ANGPT2). While ANGPT1 permits maturation or stabilization of blood vessels through TIE2, ANGPT2 blocks this pathway causing remodeling of vascular sprouts subsequent to exposure to VEGF[149]. High serum levels of ANGPT2 have been related to poor response to bevacizumab treatment[150], suggesting that ANGPT2 has a critical role in the resistance mechanism against anti-VEGF therapy[151].

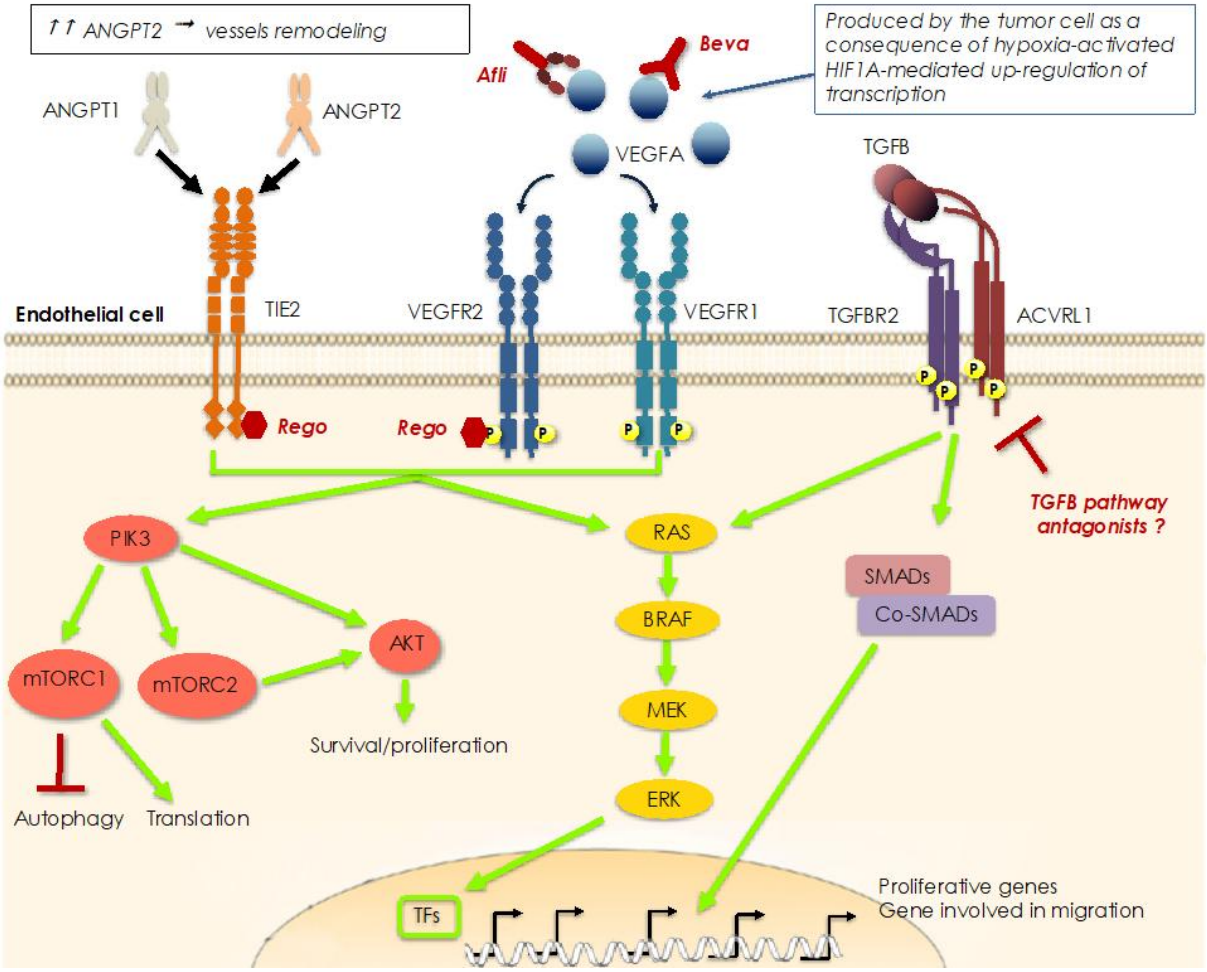

Figure 3. Mechanisms of resistance to anti-angiogenic therapy. Hyperproduction of VEGFA is a consequence of the hypoxic status in the center of the tumor which activates HIF1A, a transcription factor that regulates VEGFA transcription. Overproduction of VEGFA can be blocked by monoclonal antibodies (bevacizumab, Beva) or a VEGF-trap (aflibercept, Afli). Alternatively, the signal can be blocked downstream of the dedicated receptor by small kinase inhibitors like regorafenib (Rego), which can also inhibit the TIE2 receptor, thus imposing a double anti-angiogenic blockade. In fact, both receptors are critical in stimulating the proliferation and migration of the endothelial cells, necessary for the formation of new vessels into and around the tumor mass. In particular, TIE2 receptor is regulated by ANGPT1 and 2, the former inducing signals involved in maturation and stabilization of blood vessels, whereas the latter blocks this signaling and triggers remodeling of vascular sprouts. Overproduction of ANGPT2 can drive a parallel neo-angiogenic signaling when VEGR-driven signaling is impeded by Beva-based therapy. In addition, another parallel neo-angiogenic pathway that can act as an escape route to VEGFA blockade is initiated by TGFB binding to a multimeric receptor formed by a TGFB2 dimer in complex with an activin receptor-like kinase 1 (ACVRL1) dimer.

In preclinical models, the double block of VEGF and ANGPT2 inhibited revascularization and tumor progression of tumors resistant to anti-VEGF therapy[152,153]. Finally, cytokines have also been involved in mediating resistance to anti-angiogenic therapy. IL-17 and its downstream factor, granulocyte colony-stimulating factor (G-CSF), are the leading players of inflammation; it has been reported that a high level of IL-17 induces cells to produce a paracrine network that confers resistance to anti-VEGF therapy. Serum G-CSF levels in CRC patients are higher than those in healthy volunteers and are associated with the stage of tumor progression[154,155].

Interestingly, a novel anti-angiogenic approach that might help in circumventing resistance to anti-angiogenic therapies could be represented by metronomic chemotherapy, a schedule where low doses of chemotherapy are administered chronically. In fact, several studies demonstrated that the administration of 5-FU under metronomic chemotherapy schedule induces an antiangiogenic effect behind the cytotoxic effect[156]. In addition, it has been reported a reasonable disease control with minimal side effects and good quality of life in elderly or heavily pre-treated patients under metronomic chemotherapy schedule. Moreover, a phase III randomized trial has shown that maintenance therapy with metronomic capecitabine plus bevacizumab following cycles of conventional treatment with capecitabine + oxaliplatin + bevacizumab significantly improved PFS compared to the observation group with no worsening of quality of life[157].

Resistance to immune checkpoint inhibitors

Compared to pMMR/microsatellite stable CRCs, dMMR/MSI-H CRCs - because of the defects in repairing DNA damage/insertion of microsatellites - present a high mutation burden and produce a plethora of immunogenic neoantigens on the MHC, thus recruiting more tumor infiltrating lymphocytes. However, dMMR/MSI-H CRCs also highly upregulate the expression of immune checkpoints - (i.e., PD-1 ligands, CTLA-4 ligand, LAG-3, and IDO1) that account for the suppression of an active immune response despite the high tumor infiltrating lymphocyte count [Figure 4][158]. Because of this scenario dMMR/MSI-H CRCs have been shown to be sensitive to treatment with ICIs in several phase 2 trials[84,158-160]. Notably, the KEYNOTE-177 phase 3 trial compared the effect of anti-PD-1 pembrolizumab *vs*. SOC chemotherapy given as a first line therapy. A significant clinical benefit of pembrolizumab resulting in the doubling of PSF was evident and 83% of pembrolizumab responders were still responding after 2 years or longer, compared to the 35% of the chemotherapy responder. However, 30% of pembrolizumab-treated patients had primary resistance[161] indicating that additional therapeutic strategies are needed in the field of immunotherapy. Interestingly, Checkmate-142 phase 2 clinical trial reported that a double targeting strategy given as a first-line therapy led to significant and clinically meaningful improvements in patients' outcomes. Specifically, anti-PD-1 nivolumab was administered together with anti-CTLA4 ipilumab, and the PSF rate at 12-month was 77% compared to 55% reported with single PD-1 blockade in the KEYNOTE-177 study[84]. At variance with patients with dMMR/MSI-H CRC those with microsatellite stable mCRC had very limited clinical benefit when receiving the combination with CTLA-4 and PD-L1 inhibitors[160]. Given that about 95% of mCRC cases are pMMR/microsatellite stable tumors and resistant to ICIs, several efforts are focused on the understanding of the mechanisms of resistance to immunotherapy for the development of new therapeutic approaches. Notably, another promising inhibitor to be added to the combination of ICIs is relatlimab, a monoclonal antibody that binds to LAG-3, a checkpoint molecule that functions to negatively regulate homeostasis of T- and B-cells and NK cells[162]. So far, it has been tested in clinical trials for treating melanomas and, interestingly, in a phase 3 trial in patients with newly diagnosed inoperable or metastatic melanomas, its combination with nivolumab doubled PSF[162]. In addition, a newly characterized monoclonal anti-LAG-3 antibody resulted in a significant delay in tumor growth when combined with PD-1 blockade in mice transplanted with colorectal cancer cells[163].These results suggest that the addition of anti-LAG-3 monoclonal antibodies might improve the response to currently used ICIs also in CRC. Finally, a brief mention about IDO1 inhibitors is due, given that this enzyme is overexpressed in CRC tissues. IDO1 catalyzes the oxidative cleavage of tryptophan to kynurein, a metabolite responsible for an immunosuppressive environment via the blockade of the activation of effector cells and the stimulation of immunosuppressive cells[164]. In particular, high levels of kynurein have been shown to predict resistance to PD-1 blockade and OS upon administration of nivolumab in advanced melanoma and renal cell carcinoma patients. Remarkably, in preclinical models IDO inhibitors had negligible effect when used alone but resulted synergic with anti-CTL-A4 and anti-PD-1 therapies in controlling cancer burden and favoring mice survival[165]. Therefore, different inhibitors are currently being studied in several tumor types as co-administered with anti-PD-1 antibodies (i.e., nivolumab or pembrolizumab) or anti-PD-L1 antibodies (i.e.,

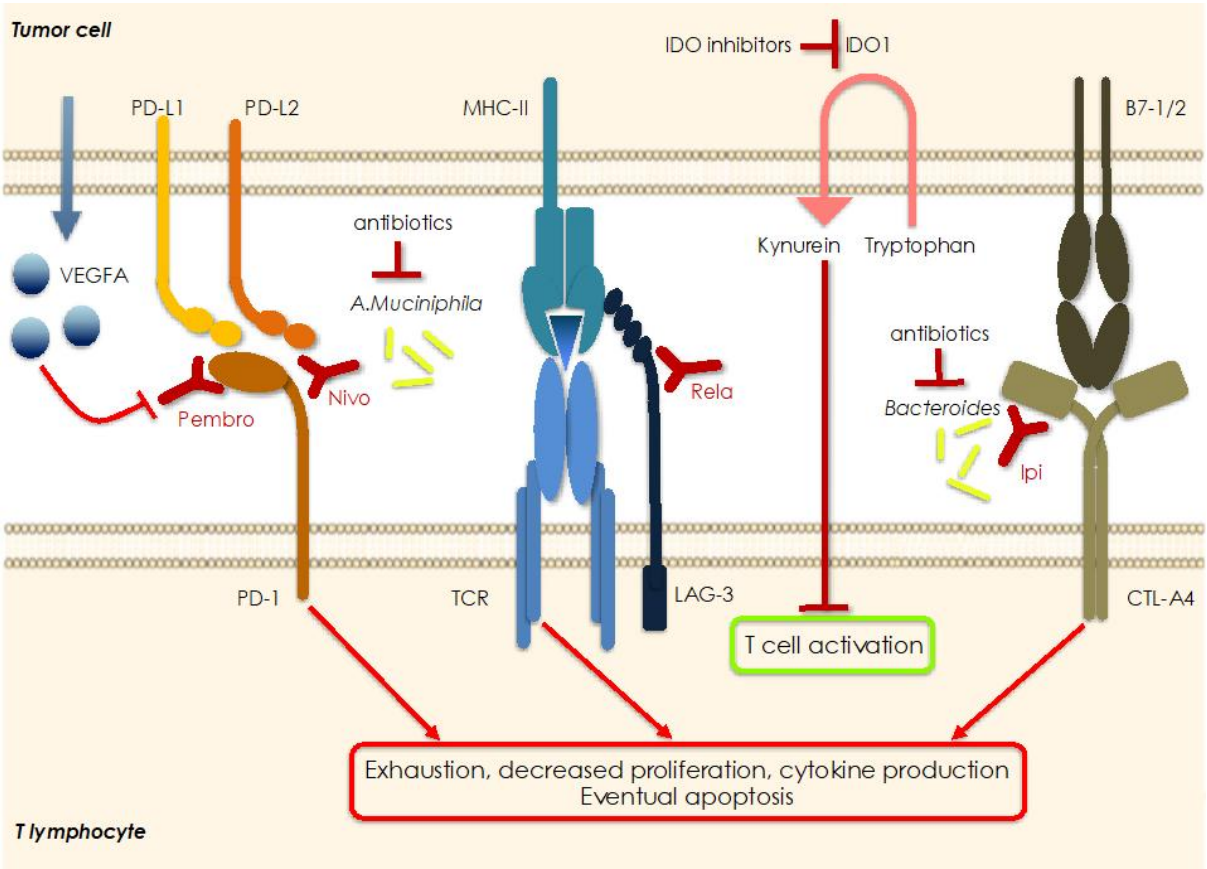

Figure 4. Mechanisms of resistance to immuno-therapy. Cancer cells can express several immunosuppressive molecules that can be blocked using specific inhibitors in order to re-activate the anti-tumor immune response. Often, they are redundant so that the re-activation of one pathway may be compensated by hyperactivation of a different immunosuppressive route so that resistance to therapy is the result. Resistance to anti-PD1 blockade may be due to overproduction and release of VEGFA, as well as by overexpression of IDO1 with consequent increased levels of kynurein in the microenvironment. Specific components of the microbiota are also necessary for the successful blockade of PD-1 and CTL-A4 indicating that antibiotics may represent a resistance factor to ICIs. Finally, concurrent blockade of two or more immune checkpoints has been shown to improve the response and overcome the resistance to ICIs. Pembro: Pembrolizumab; Nivo: nivolumab; Rela: relatlimab; Ipi: ipilumab; IDO1: indoleamine 2, 3-dioxygenase 1.

atezolizumab or durvalumab)[166] and the same approach is to be envisaged for ICIs-resistant CRCs.

An immunosuppressive role has been reported for VEGF, which can act via different mechanisms such as by decreasing the number of TILs and activating immune checkpoint molecules[167]. Notably, in a CRC mouse model it has been reported that addition of anti-angiogenic therapy reduced intrinsic resistance to an anti-PD-1 antibody via normalizing tumor vascularization and reducing hypoxia[168]. Several trials are ongoing to evaluate the efficacy of combining ICIs with an anti-angiogenic strategy plus chemotherapy, being the molecules tested bevacizumab and regorafenib.

Recently, several studies have shown that the gut microbiota plays a key role in regulating the efficacy of ICIs for cancer treatment of different solid tumors [Table 3, Figure 4][169]. For example, the treatment with an anti-CTLA-4 monoclonal antibody controlled the growth of different tumor models (including a CRC model) in mice maintained in specific pathogen-free conditions and in the presence of specific *Bacteroides*, but not in germ-free mice and mice previously treated with antibiotics. Therefore, it appears that the gut microbiota might regulate the efficacy of immunotherapy with anti-CTLA-4. Accordingly, the introduction

of two specific *Bacteroides* species (i.e., *B. thetaiotaomicron* and *B. fragilis*) in germ-free mice and antibiotics-treated mice restored the response to anti-CTLA-4 treatment[170]. Notably, a series of studies in melanoma mouse models and patients uncovered other microbiota components as instrumental in determining the response to ICIs. For example, in a melanoma mouse model the addition of the commensal gut *Bifidobacterium* to PD-L1 checkpoint blockade nearly abolished the growth of scarcely sensitive tumors[171]. Comparative analysis of the oral and gut microbiome of melanoma patients undergoing anti-PD-1 immunotherapy revealed significant differences in the diversity and composition of the microbiome of responders *vs.* non-responders. Similarly, the analysis of patient fecal microbiome samples showed significant diversity and relative abundance of bacteria of the Ruminococcaceae family in responding patients[172]. Analysis of baseline stool samples from metastatic melanoma patients before immunotherapy uncovered significant association between commensal microbial composition and clinical response. Bacterial species more abundant in responders included *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium*. Accordingly, in a mouse model fecal microbiota transplantation from responding patients in germ-free mice improved tumor control, augmented T cell responses, and increased efficacy of anti-PD-L1 therapy[173]. Routy *et al.*[174] showed that fecal microbiota transplantation from cancer patients who responded to ICIs into germ-free or antibiotic-treated mice improved the efficacy of anti-PD-1 therapy, whereas fecal microbiota transplantation from non-responding patients was ineffective. Profiling patients' stool samples revealed low levels of the bacterium *Akkermansia muciniphila* in non-responding patients with lung and kidney cancer. Accordingly, oral supplementation of the bacteria after fecal microbiota transplantation with non-responders feces in antibiotic-treated mice restored the response to PD-1 blockade[174]. Regulation of gut microbiota composition or addition of the "right" bacteria to therapy seems therefore a very promising approach to overcome resistance, or at least significantly improve the response to ICIs.

CONCLUSION

Intrinsic and acquired resistance to chemo-, targeted, and immune-therapy remain the core problem(s) to tackle for ameliorating the outcome of CRC patients, especially in the metastatic setting. Significant improvements and longer expectancy of life have been reached using a combination/integration of the different types of therapy, but only the identification of additional targetable liabilities to be exploited in specific settings will allow to concomitantly attack the tumor from different angles, hopefully leading to its eradication. In this sense, promising approaches are: the development of novel nanoparticles able to vehicle chemotherapy directly inside the cells avoiding the intake/efflux systems, often deregulated in resistant cells; the induction of synthetic lethality via the use of inhibitors of specific DNA damage checkpoints or DNA repair enzymes in tumors presenting defects in the DNA repair mechanisms; the use of BH-3 mimetics in tumors highly expressing anti-apoptotic members of the BCL2 family; the targeting of several kinases shown to sustain resistance to classic chemotherapy and also resistance to target therapy via activation of feed-back loops; the targeting of pathways crucial for cancer stem cells maintenance and viability; the targeting of several components of the TME, recognized to be responsible for an immune-privileged environment, among which the so-called immune checkpoints; and the modulation of the microbiota. For the most of these approaches an unavoidable task will be patient stratification according to specific biomarkers and/or genetic defects, indicating that precision medicine is the ultimate path to cover in order to identify effective therapies for each patient. In addition, given the high tumoral heterogenicity and the Darwinian pressure exerted by anti-cancer treatments, to prevent and/or overcome resistance it will be also essential to combine different types of therapies and using vertical approaches to target multiple nodes along the same pathway.

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Authors' contributions

Drafting the article, revising it, and giving final approval prior to submission: Grassilli E, Cerrito MG

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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HDAC inhibitors with potential to overcome drug resistance in castration-resistant prostate cancer

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Abstract

Epigenetic mechanisms play an important role in the development and persistence of cancer, and histone deacetylase (HDAC) inhibitors are promising anticancer drugs targeting epigenetic modes. Efficient anticancer drugs for the treatment of castration-resistant prostate cancer (CRPC) are sought, and approved HDAC inhibitors have shown promising results on the one hand and severe drawbacks on the other hand. Hence, ways to break the drug resistance mechanisms of existing HDAC inhibitors as well as the design of new promising HDAC inhibitors which can overcome the disadvantages of the classic HDAC inhibitors are of great importance. In this work, HDAC inhibitors with the potential to become a mainstay for the treatment of CRPC in the future as well as suitable combination treatments of HDAC inhibitors with other anticancer drugs leading to considerable synergistic effects in treated CRPCs are discussed.

Keywords: Histone deacetylases, HDAC inhibitors, castration-resistant prostate cancer, drug resistance

INTRODUCTION

More than 1.2 million new cases of prostate cancer cases are reported per year worldwide (2018) rendering prostate cancer the second most diagnosed cancer in men along with a rising incidence over the last years[1]. The formation of metastases dramatically reduces the survival rates in prostate cancer patients[2]. Advanced

forms of prostate cancer are commonly treated by hormone therapy, either by surgical castration or by chemotherapy targeting hormone release[3]. However, patients develop castration-resistant prostate cancer (CRPC) forms within a median time of two years after treatment, which pose a life-threatening danger to affected patients with distinctly poorer prognosis[4]. CRPC can occur in metastatic (mCRPC) and non-metastatic (M0CRPC) forms. Clinical trials using anti-androgen therapies with drugs such as apalutamide, enzalutamide, or darolutamide showed promising outcomes and significantly prolonged metastasis-free survival in M0CRPC patients[5-7]. Treatment options for mCRPC patients include taxanes such as docetaxel and cabazitaxel as well as various anti-androgens (bicalutamide and enzalutamide)[8]. The development of the selective CYP17 inhibitor abiraterone and its prodrug abiraterone acetate, specifically blocking androgen synthesis and androgen receptors via CYP17 enzyme inhibition, has led to another valuable first-line treatment option for mCRPC patients[9].

Chromatin remodeling is a crucial mechanism in proliferating cells and affects transcription, replication, and DNA repair processes. The nucleosome is the basic unit of chromatin and consists of the positively charged histone core octamer proteins (H2A, H2B, H3, and H4, each twice per octamer) wrapped by DNA of 147 base pairs and one attached H1 histone[10,11]. *N*-terminal lysines of the histones are crucial for their DNA interaction, and the charge of these lysines is regulated by cellular *N*-acetylation processes. Acetylation of histones reduces their interaction with DNA leading to chromatin decondensation and gene transcription, while histone deacetylation silences gene expression in the densely packed chromatin[12]. Histone modifying enzymes such as histone acetyl transferases (HATs), which catalyze lysine acetylation, and histone deacetylases (HDACs), which catalyze the removal of acetyl groups from target protein lysines, play an eminent role in these processes and, in particular, inhibitors of HDACs were developed as anticancer agents[13]. The scope of HDAC inhibitors is promising since they also affect the acetylation state of non-histone proteins, such as p53, Hsp90, STAT3, and NF-κB, and regulate the stability and/or DNA binding properties of these non-histones in this way with significant effects on, for instance, gene transcription, cell division, signal transduction, DNA repair, and apoptosis induction[14,15]. In terms of HDACs and prostate cancers, the HDAC-based regulation of the androgen receptor (AR) via the acetylation state of Hsp90 is especially interesting and of high relevance for the design of HDAC inhibitor-based prostate cancer therapies for patients with poor prognosis (see below)[15,16]. *N*-Methylation of lysines is another regulatory modification of histones controlled by lysine demethylases (KDMs), which can have silencing and activating effects on gene transcription in cancers[17]. KDMA1 (LSD1) has oncogenic functions in prostate cancers by AR co-activation, suppression of p53, and activation of c-Myc expression, and the development of KDM inhibitors as anticancer agents (e.g., cyclopropylamines) paves the way to promising epigenetic treatment options. The combination of KDM1A and HDAC inhibitors showed synergistic effects on glioblastoma cells[17]. The HDAC inhibitor vorinostat also inhibited EZ2H and H3K4 demethylases in the micromolar concentration range[17-19].

A few HDAC inhibitors have already reached clinical application and are used for the treatment of multiple myeloma (panobinostat) and T-cell lymphoma (vorinostat, romidepsin, and belinostat); however, the performance of these first-generation HDAC inhibitors in solid tumors is rather poor[20]. Nevertheless, HDACs can be suitable anti-prostate cancer drug targets. HDAC1, for instance, was found to be upregulated in hormone refractory prostate cancer[21]. HDAC1, -2, and -3 expression was associated with Ki-67-positive prostate cancer cell fractions, and high HDAC2 levels were detected in patients with reduced disease-free survival[22]. HDACs are necessary for functional AR signaling in prostate cancers, and HDAC inhibitors such as vorinostat and panobinostat blocked AR function by reducing AR expression and inhibiting coactivator/RNA polymerase II complex formation after AR binding to its DNA target element[23]. Resistance mechanisms of prostate cancers to HDAC inhibitor treatment comprise the

upregulation of detoxifying P-glycoprotein (P-gp) transporters, increased expression of HDAC enzymes, suppression of HAT enzymes, and upregulation of tumorigenic p21 (p21 is usually a tumor suppressor, but oncogenic functions of p21 were reported upon HDAC treatment)[24]. HDAC inhibitors also induced epithelial-to-mesenchymal transition (EMT) in prostate cancers, which might be another reason for the drawbacks of HDAC inhibitors in solid tumors[25].

HDAC enzymes can be subdivided into four classes. Classes I (HDAC1, -2, -3, -8), IIa (HDAC4, -5, -7, and -9), IIb (HDAC6 and -10), and IV (HDAC11) are metal-dependent with a catalytic zinc ion in the active site, while Class III HDACs (sirtuins) are not dependent on metals[26,27]. Due to the catalytic zinc ion of Class I, II, and IV HDACs, many HDAC inhibitors have a molecular zinc-binding group (ZBG) such as carboxylates, hydroxamic acids, benzamides, substituted ketones, mercaptoacetamides, or depsipeptides[28]. The modular composition of most HDAC inhibitors comprising a ZBG and a capping group connected by a linker allows the fine-tuning of HDAC inhibitors in terms of pharmacokinetics and anticancer activity[28]. Several dual or multimodal HDAC inhibitors have been reported, which showed improved anticancer activities especially against solid tumors[29,30]. This development is still ongoing and offers interesting compounds for the treatment of drug-resistant CRPC where first-generation HDAC inhibitors failed to perform.

This review provides the current state of the art of HDAC inhibitors under investigation in CRPCs and the potential of HDAC inhibitors to overcome drug resistance in this severe cancer form. A focus is set on next generation HDAC inhibitors with improved biological properties.

HDAC INHIBITORS IN CRPC CLINICAL TRIALS

Several clinical trials with HDAC inhibitors were carried out to monitor advantages and problems of the usage of HDAC inhibitors in the clinics. The clinical trials of HDAC inhibitors for the treatment of castration-resistant prostate cancer diseases were recently reviewed, and only the main outcomes are summarized in Table 1 and Figure 1[24,31].

The depsipeptide romidepsin underwent a phase 2 clinical trial with 35 CRPC patients. Intravenous romidepsin treatment (13 mg/m^2) led to radiological partial response in two patients and stable disease in 11 patients, while 22 patients showed progressive disease. Although no grade 4 toxicities were observed, 11 patients had to abandon the trial early, and, thus, romidepsin was not recommended for further phase 3 trials for CRPC[32].

The hydroxamic acid vorinostat (400 mg/day, p.o.) showed drug-induced toxicities in 11 out of 27 CRPC patients during a phase 2 trial, who had to be removed from the study, while its anticancer activity was poor: only 2 patients had stable disease and 13 patients showed disease progression[33]. It seems that high IL-6 levels contributed to the failure of vorinostat in the patients with progressive CRPC disease.

Similar to romidepsin, the hydroxamate derivative panobinostat underwent a phase 2 trial with 35 CRPC patients. Intravenous panobinostat administration (20 mg/m^2) led to PSA (prostate-specific antigen) reduction in only 14% of the patients and disease progression in 29 out of 35 patients[34]. A phase 1 study of orally administered panobinostat compared with oral panobinostat in combination with docetaxel and prednisone showed only PSA reduction effects in five out of eight CRPC patients of the combination arm of the trial[35]. The oral panobinostat monotherapy showed no effects, and, thus, future studies should include combination therapies with suitable anticancer drugs. A combination study with oral panobinostat and bicalutamide (phase 1/2 trial) in nine CRPC patients led to stable PSA levels in three patients and to PSA

Table 1. CRPC clinical trials of HDAC inhibitors

Drug(s)	Trial	*n*	Dosage	Outcome
Romidepsin[32]	Phase 2	35	30 mg/m^2 (i.v.)	PR = 2; SD = 11; PD = 22
Vorinostat[33]	Phase 2	27	400 mg/day (p.o.)	SD = 2; PD = 13
Panobinostat[34]	Phase 2	35	20 mg/m^2 (i.v.)	SD = 4; PSA reduction = 5; PD = 29
Panobinostat (+ Docetaxel and Prednisone)[35]	Phase 1	8	15 or 20 mg 3× per week (p.o.)	PSA reduction = 5
Panobinostat (+ Bicalutamide)[36]	Phase 1	9	60, 90, or 120 mg/week (p.o.)	Stable PSA = 3; PSA reduction = 4
Panobinostat (+ Bicalutamide)[37]	Phase 2	29	40 mg triweekly for 2 weeks (p.o.)	rPF = 47.5%; median time to rP = 33.9 weeks
Pracinostat[38]	Phase 2	32	60 mg, 3× per week (p.o.)	SD = 7; PSA reduction = 2

PR: Partial response; SD: stable disease; PD: progressing disease; PSA: prostate-specific antigen; rP: radiographic progression; rPF: radiographic progression-free.

reduction of more than 50% in two patients[36]. The phase 2 trial of oral panobinostat (40 mg triweekly for two weeks) with oral bicalutamide showed promising results in CRPC patients, who were resistant to second-line antiandrogen therapy (2ndLAARx)[37]. This combination treatment was well tolerated and led to a distinct number of radiographic progression-free patients (47.5%) and prolonged median time to radiographic progression (rP) in treated CRPC patients (33.9 weeks). In addition, five patients showed PSA decline of more than 30%. The results indicate that panobinostat plus bicalutamide overcomes androgen resistance in a considerable number of CRPC patients. It is remarkable that panobinostat was less toxic to patients than other HDAC inhibitors such as romidepsin and vorinostat.

Pracinostat, which has received orphan drug status by the FDA for the treatment of AML, was also studied in a clinical phase 2 trial with 32 CRPC patients. Although only two patients displayed PSA reductions of more than 50%, the drug (60 mg, 3× per week, p.o.) was well tolerated, led to stable disease in 22% of the patients, and reduced the number of circulating tumor cells in nine patients[38].

In brief, the cinnamoyl derivatives panobinostat and pracinostat showed improved toxicity profiles and led to certain antitumor responses in CRPC patients, in particular, when combined with other drugs such as docetaxel or bicalutamide (in the case of panobinostat). In contrast, vorinostat and romidepsin showed drug-induced toxicities in a considerable number of patients who had to stop treatment with these drugs because of them. The relatively meager responses caused by the mentioned first-generation HDAC inhibitors in CRPC patients when applied as a monotherapy is the reason why no phase 3 studies in CRPC patients have been completed for these drugs until today.

STRATEGIES TO IMPROVE HDAC INHIBITOR ACTIVITIES IN CRPC

To overcome the clinical drawbacks of the first-generation HDAC inhibitors, a thorough elucidation of HDAC inhibitor mechanisms apart from or in consequence of HDAC inhibition is necessary, in particular, in combination with other anticancer drugs. In addition, the development of new tuned HDAC inhibitors appears to be promising in terms of improved clinical outcome. The most relevant research outcomes are provided below.

HDAC inhibitor mechanisms beyond HDAC

HDAC inhibition can lead to certain downstream effects in prostate cancer cells [Figure 2, Table 2]. The chemical structures of the investigated compounds are shown in Figure 1. The endogenous HDAC1 inhibitor protein maspin (mammary serine protease inhibitor), which was induced by the HDAC inhibitor

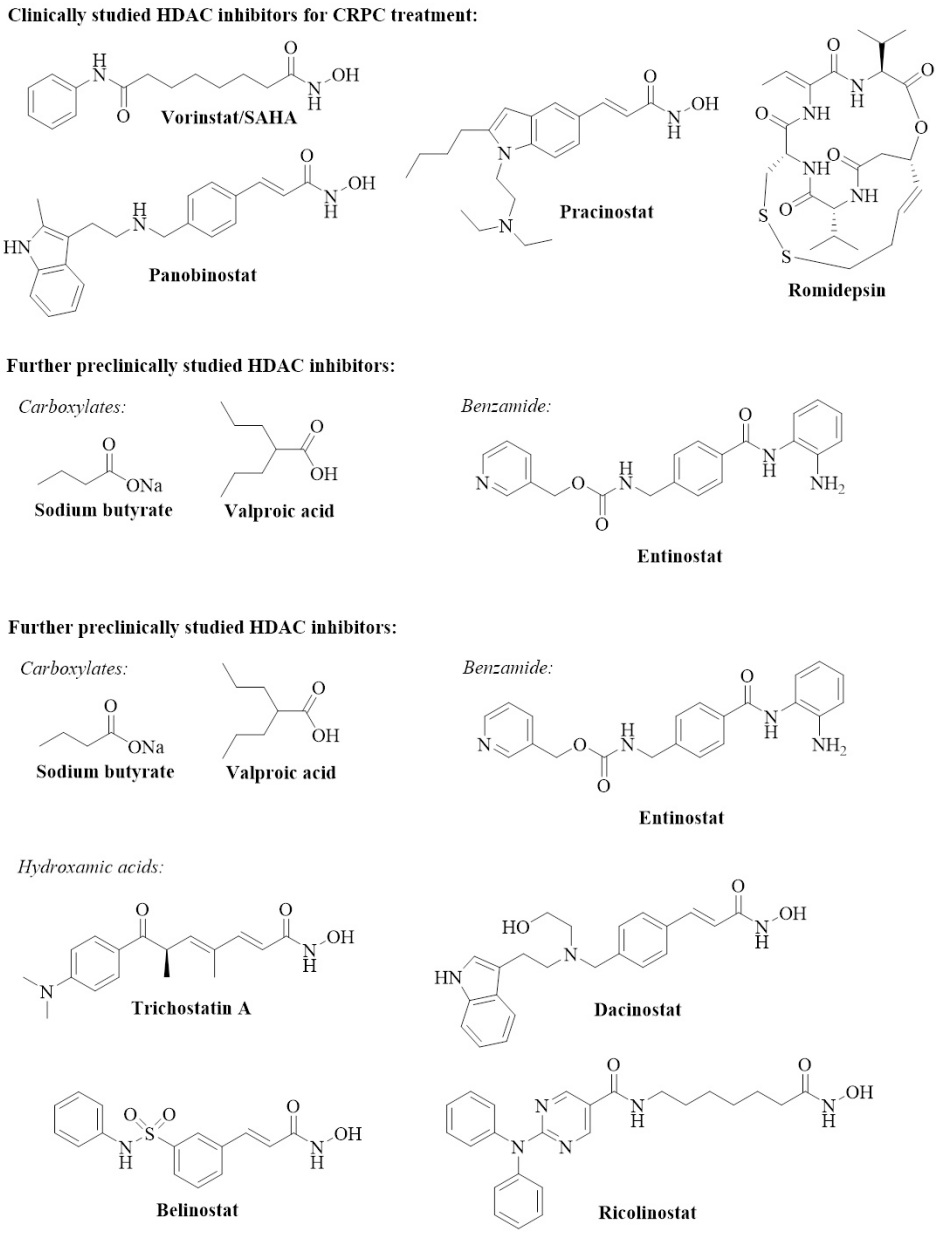

Figure 1. Structures of clinically and preclinically tested HDAC inhibitors. HDAC: Histone deacetylase; CRPC: castration-resistant prostate cancer.

entinostat, suppressed AR expression in prostate cancers and led to sensitization of androgen-sensitive LNCaP and castration-resistant 22Rv1 prostate cancer cells to enzalutamide treatment[39].

Acetylation of Hsp90 is regulated by cytoplasmic HDACs such as HDAC6, while Hsp90 regulates AR activity and stability in prostate cancers[40]. Hormone treatment induced castration-resistant phenotype and upregulated HDAC6 expression in prostate cancer cells. Belinostat (PXD101) suppressed Hsp90 activity, GSK-3β, and AR function by HDAC6 inhibition and prevented the development of castration-resistant phenotype in prostate cancer cells. In addition, belinostat (40 mg/kg bid, i.p.) showed improved activities in 22Rv1 CRPC xenograft models in combination with the androgen antagonist bicalutamide (50 mg/kg, p.o.)[41]. Dacinostat (LAQ824), a close analog of panobinostat, suppressed the Hsp90 client

Table 2. HDAC inhibitor effects beyond HDAC inhibition in prostate cancers

HDAC inhibitor	Effect(s)	Cell lines/tumor models
Belinostat[41]	Hsp90, AR, and GSK-3β suppression	LNCaP, C4-2B, 22Rv1
Dacinostat[42]	AR degradation by high acetyl-Hsp90, Akt inactivation	LNCaP
Entinostat[39]	AR suppression, enzalutamide sensitization	LNCaP, 22Rv1
Entinostat[43]	IFNγ production, suppressed Foxp3, upregulated acetyl-STAT3, increased survivin vaccine activity	CR Myc-CaP
Panobinostat[46]	HMGA2 suppression, EMT formation, increase of acetyl-p53 and acetyl-AR	PKV, MES-like cells/tumors
Trichostatin A[44]	Upregulated FGF8 and NF-κB	PC3M
Trichostatin A and sodium butyrate[47]	Upregulated CD133	Prostate cancer derived primary endothelial cultures

Hsp90: Heat shock protein 90; AR: androgen receptor; IFNγ: interferon-γ; STAT3: signal transducer and activator of transcription 3; CR: castration-resistant; HMGA2: high mobility group AT-hook 2; EMT: epithelial-to-mesenchymal transition; MES: mesenchymal; PC3M: metastatic PC3 cells; PKV: *Pten*$^{L/L}$, *Kras*$^{G12D/+}$, *Vim-GFP*.

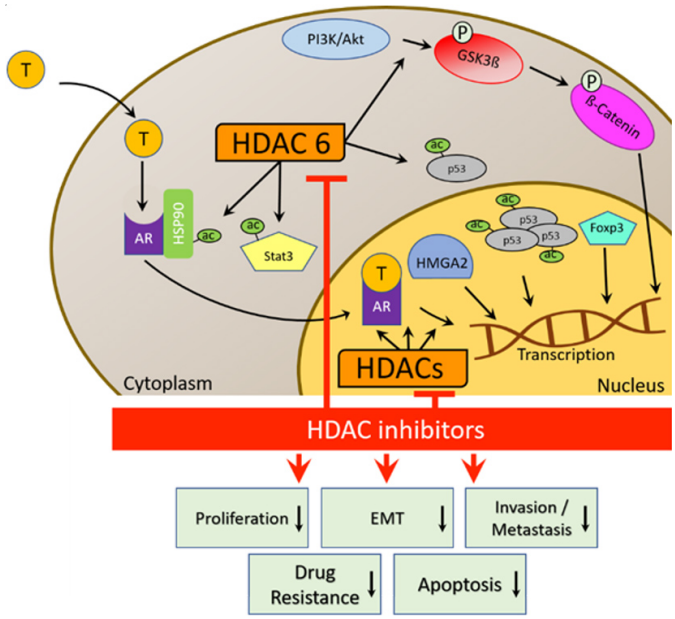

Figure 2. HDAC network and cellular effects of HDAC inhibitors in CRPC. HDAC: Histone deacetylase; HDACi: HDAC inhibitor; T: testosterone; Hsp90: heat shock protein 90; AR: androgen receptor; STAT3: signal transducer and activator of transcription 3; HMGA2: high mobility group AT-hook 2; EMT: epithelial-to-mesenchymal transition.

protein AR in LNCaP prostate cancer cells by an increase of acetylated Hsp90 levels accompanied by Hsp90-AR dissociation and AR decomposition[42].

Dacinostat also inactivated Akt by disruption of HDAC-PP1 (protein phosphatase 1) complexes, leading to the dephosphorylation of Akt proteins in PC3 prostate cancer cells[42]. Consequently, factors associated with Akt signaling were regulated by HDAC inhibitors. The Class I HDAC inhibitor entinostat (5 mg/kg/day, 5 days per week, p.o.) augmented distinctly the anticancer activity of the survivin peptide vaccine SurVaxM as well as IFNγ (interferon-γ) production in Myc-expressing CRPC mouse models (CR Myc-CaP). Entinostat upregulated acetyl-STAT3 accompanied by suppression of Foxp3 in cancer cells, which was suggested as its possible mode of action[43]. As part of its hormone-independent prostate cancer promoting effect, the natural HDAC inhibitor trichostatin A (TSA) increased the transcriptional activity of NF-κB in PC3M

prostate cancer cells associated with upregulation of fibrose growth factor FGF8[44]. Thus, NF-κB suppressing agents might be promising combination partners of entinostat in terms of CRPC eradication. The PI3K/Akt signaling pathway is also involved in EMT induction[45]. EMT processes upregulate HMGA2. HMGA2 is a protein crucial for the regulation of gene expression by binding to AT-rich regions of DNA, which is the site where a replication complex is formed and the DNA synthesis is initiated. Interestingly, panobinostat was shown to suppress HMGA2 associated with reduced EMT formation and cell stemness. In addition, panobinostat increased the acetyl-p53 and acetyl-AR levels and prevented mCRPC formation *in vivo* at a dose of 10 mg/kg for five days per week[46]. In terms of cancer stem cell markers, the HDAC inhibitors TSA and sodium butyrate upregulated the expression of the surface stem cell marker CD133 in prostate cancer derived primary epithelial cultures, which was based on chromatin relaxation leading to gene expression[47].

Promising combinations of HDAC inhibitors with anticancer drugs

The detailed knowledge of the mechanisms of action of HDAC inhibitors in prostate cancers can be applied for combination therapies together with other suitable drugs against prostate cancer to achieve improved anticancer responses [Table 3, Figures 1 and 3]. DNA-based mechanisms appear to be promising drug targets for combination partners of HDAC inhibitors. Inhibition of DNA methylation was positively correlated with HDAC inhibition and sodium butyrate in combination with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine significantly enhanced histone H4 acetylation and induced AR gene re-expression in androgen-independent DU145 prostate cancer cells. Moreover, it led to G2/M cell cycle arrest and reduced toxicity to non-malignant cells[48]. More recently, panobinostat was investigated in combination with the DNA methylation inhibitor hydralazine in various prostate cancer cells. The combination therapy was particularly active against androgen-independent DU145 prostate cancer cells, induced apoptosis, and reduced colony formation, invasion, and migration[49]. Hence, the combination of panobinostat with hydralazine has the potential to block prostate cancer progression and might be a suitable therapy in future clinical studies.

Poly-ADP-ribosylpolymerase (PARP) is an enzyme involved in DNA repair. And the PARP inhibitor olaparib performed well in advanced clinical trials with CRPC patients who have a BRCA mutation, which led to the approval of olaparib for the treatment of BRCA-mutant prostate cancer[50,51]. The combination of vorinostat with olaparib synergistically reduced prostate cancer cell viability, induced apoptosis in DU145 and PC3 prostate carcinoma cells, and suppressed DNA repair as well as DNA repair protein expression (BRCA1 and RAD51) in DU145 cells[52]. The combination of vorinostat with the PARP inhibitor veliparib also revealed synergistic effects on prostate cancer cell viability (in particular, on the viability of BRCA1-mutant DU145 cells), apoptosis induction, DNA damage production, and BRCA1 suppression. The vorinostat/veliparib combination therapy suppressed BRCA1 by downregulation of the UHRF1 protein, which forms a stable DNA repair protein complex with BRCA1, and suppression of UHRF1 led to degradation of BRCA1[53]. Thus, the combination of vorinostat with PARP inhibitors such as olaparib and veliparib appears to be a promising combination therapy for future clinical trials with CRPC patients.

Vorinostat was also studied in combination with the microtubule-stabilizing anticancer taxane drug docetaxel, and the combination showed synergistic growth inhibitory effects on 22Rv1 and VCaP CRPC cells. This combination therapy suppressed AR and Bcl-2 expression, AR translocation into the prostate cancer cell nucleus, and AR signaling. The reduced accumulation of the AR in the nucleus was based on increased acetylation and bundling of tubulin in cells treated with the HDAC inhibitor vorinostat and the microtubule stabilizer docetaxel[54]. Another study targeting AR signaling comprised the combination treatment of androgen-accustomed DuCaP-N prostate cancer cells with the HDAC inhibitor TSA, the anti-androgen bicalutamide, and the 5α-reductase inhibitor finasteride. The combination of TSA with

Table 3. HDAC inhibitors in combination with other drugs against CRPC

HDAC inhibitor	Combination partner (function)	Outcome
Sodium butyrate[48]	5-Aza-2′-deoxycytidine (DNA methyltransferase inhibitor)	Enhanced histone H4 acetylation, induced AR gene re-expression, G2/M cell cycle arrest, reduced non-malignant cell toxicity
Panobinostat[49]	Hydralazine (DNA methylation inhibitor)	induced apoptosis, reduced colony formation, invasion, and migration
Vorinostat[52]	Olaparib (PARP inhibitor)	Synergistic reduction of cell viability, induced apoptosis, suppressed DNA repair, BRCA1 and RAD51 expression downregulated
Vorinostat[53]	Veliparib (PARP inhibitor)	Synergistic effects on cell viability (BRCA1-mutant DU145), apoptosis induction, DNA damage production, BRCA1 suppression/degradation, suppressed UHRF1
Vorinostat[54]	Docetaxel (microtubules stabilizer)	Increased tubulin acetylation and bundling, suppressed AR and Bcl-2 expression, suppressed nuclear AR translocation and AR signaling
Trichostatin A[55]	Bicalutamide (anti-androgen) and finasteride (5α-reductase inhibitor)	Synergistic apoptosis induction
Panobinostat[57]	Dovitinib (multi-RTK inhibitor)	No improved effects
Ricolinostat[58]	Selumetinib (MEK inhibitor) and paclitaxel (microtubules stabilizer)	Synergistic growth inhibition and apoptosis induction, suppression of KLK2 and DUSP1, increased AR accumulation in cytoplasm
Valproic acid[62-64]	Everolimus (mTOR inhibitor) and IFNα	Increased cell growth inhibition, suppression of EGFR (epidermal growth factor receptor), ERK1, and ERK2, increased acetyl-H3
Panobinostat[67]	Everolimus (mTOR inhibitor)	Myc-CaP cell growth inhibition, suppressed clonogenic survival and G0/G1 cell cycle arrest, increased p21 and p27 expression; *in vivo* suppression of tumor growth, AR, HIF-1α, miR-20a, miR-21
Romidepsin, Entinostat[70]	Adenoviral TRAIL gene therapy	Restored CAR surface expression, augmented TRAIL-mediated caspase activity

PARP: Poly-ADP-ribosyl-polymerase; RTK: receptor tyrosine kinase; MEK: MAPK/ERK kinase; IFNα: interferon-α; mTOR: mammalian target of rapamycin; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; HIF: hypoxia inducible factor; miR: microRNA; CAR: coxsackie adenovirus receptor; TRAIL: TNF-related apoptosis-inducing ligand.

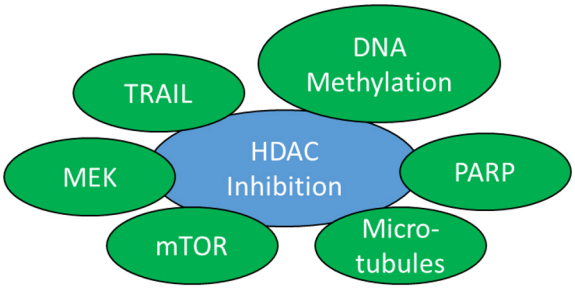

Figure 3. Suitable targets for combination therapies with HDAC inhibitors in CRPC. HDAC: Histone deacetylase; CRPC: castration-resistant prostate cancer; MEK: MAPK/ERK kinase; TRAIL: TNF-related apoptosis-inducing ligand.

bicalutamide and finasteride led to synergistic apoptosis induction in the DuCaP-N cells[55].

Protein kinases are valuable targets for new anticancer drugs. Enhanced receptor tyrosine kinase (RTK) signaling was observed in advanced prostate cancers and appeared as a suitable drug target for targeted therapies[56]. Thus, the combination of HDAC inhibitors with TK inhibitors seems to be reasonable. However, the combination of the multi-RTK inhibitor dovitinib (TKI258) with panobinostat did not show improved anticancer effects on prostate cancer cells since dovitinib appeared to be particularly inactive in the tested prostate cancer cells[57]. MAPK/ERK kinase (MEK, a serine/threonine/tyrosine kinase) inhibitors suppress the Ras-MAPK signaling pathway in cancers, and a series of anticancer active MEK inhibitors was developed[58]. The MEK inhibitor selumetinib was studied in combination with the HDAC6 inhibitor ricolinostat in various CRPC cell lines (PC3, DU145, and 22Rv1). While the combination treatment revealed

synergistic effects in all three cell lines, 22Rv1 cells were particularly sensitive in terms of growth inhibition. A suppression of the AR target genes KLK2 and DUSP1 was observed in treated 22Rv1 cells as well as increased AR accumulation in the cytoplasm. Addition of the taxane paclitaxel to the ricolinostat/selumetinib combination led to synergistic growth inhibition and increased apoptosis induction in all three prostate cancer cell lines, which coincides well with the aforementioned anti-prostate cancer effects of the combined vorinostat/docetaxel treatment[59].

Akt and its downstream factor mTOR (mammalian target of rapamycin) are well established anticancer targets of the phosphatidylinositol 3-kinase (PI3K) signaling pathway[60]. Various mTOR inhibitors such as rapamycin, everolimus, and temsirolimus were developed and approved by the FDA[61]. The anticancer activities comprising inhibition of prostate cancer cell growth, migration, and invasion of HDAC inhibitors such as valproic acid were increased when combined with everolimus[62,63]. Furthermore, the activity of valproic acid plus everolimus was augmented by addition of low-dose interferon-α (IFNα), and this triple-drug therapy was superior to single-drug therapy in terms of prostate tumor cell growth inhibition and dissemination. Intracellular signaling of PC3 cells was downregulated based on suppression of EGFR (epidermal growth factor receptor), ERK1, and ERK2, while acetyl-H3 levels were increased in treated cells[64]. Panobinostat was also studied in combination with everolimus in prostate cancer. For this reason, the *c*-Myc expressing Myc-CaP prostate cancer cell line was applied because c-Myc expression reduced the activity of the mTOR inhibitor rapamycin in prostate cancer[65,66]. The combination of panobinostat with everolimus exhibited significant Myc-CaP cell growth inhibition, while the combined treatment of Myc-CaP cells at non-cytotoxic doses of 10 nM for each compound suppressed clonogenic survival and induced G0/G1 cell cycle arrest associated with increased expression of the cyclin-dependent kinase inhibitors p21 and p27. In addition, combined panobinostat (10 mg/kg, i.p.) and everolimus (10 mg/kg, p.o.) treatment for 15 days (QD × 7 schedule) showed reduction of tumor proliferation and tumor volume in mice bearing androgen-sensitive as well as castration-resistant Myc-CaP tumors. The combination treatment blocked AR and HIF-1α transcriptional activities both *in vitro* and *in vivo* and downregulated the oncogenic microRNAs (oncomirs) miR-20a and miR-21 *in vivo*, which were associated with AR/hypoxia and *c*-Myc/hypoxia signaling pathways[67].

Virus therapies have become interesting tools for gene therapies of tumor diseases and adenoviral TRAIL gene therapy caused signs of apoptosis in the examined prostates of prostate cancer patients[68]. Since gene suppression in cancer cells can be reversed by HDAC inhibitors, the combination of gene therapy with HDAC inhibitors seems to be feasible. The HDAC inhibitors romidepsin and MS-275 increased the effects of adenoviral TRAIL gene therapy on castration-sensitive LNCaP prostate cancer cells without toxicity to non-malignant prostate epithelial cells[69]. Based on this finding, the combination of TRAIL gene therapy with romidepsin or MS-275 in C4-2B CRPC cells was studied. Decreased coxsackie and adenovirus receptor (CAR) expression was correlated with increased tumorigenicity and metastasis formation in the LNCaP-derived human prostate cancer subline C4-2B, however, both aforementioned HDAC inhibitors restored CAR surface expression and augmented TRAIL-mediated caspase activity based on enhanced adenoviral transduction efficacy when combined with TRAIL gene therapy[70].

New HDAC inhibitors

Aside from combination of approved HDAC inhibitors with other anticancer drugs, the development of new HDAC inhibitors with improved anticancer properties turned out to be a reasonable strategy to tackle CRPC [Table 4, Figure 4].

Table 4. New HDAC inhibitors with promising anti-CRPC activities

Compd.	Structural motif(s)	*In vitro* activity	*In vivo* activity
CN133[71]	Hydroxamic acid, adamantyl cap	Inhibition of HDAC1-3; 100 times more active than SAHA (22Rv1 cells), inhibition of cell migration, invasion and AR signaling	Tumor growth and weight reduction by 50% (22Rv1)
2-75[74,75]	Hydroxamic acid, AR-targeting enzalutamide-type cap	HDAC inhibitory activity, induced p21, higher acetyl-tubulin levels (based on stronger HDAC6 inhibition) than SAHA, suppressed Hsp90 and AR/AR-V7	Improved long-term tumor growth inhibition, enhanced apoptosis, reduced nuclear AR accumulation (LNCaP)
CUDC-101[77]	Hydroxamic acid, EGFR/HER2-targeting erlotinib-type anilinoquinazoline cap	Suppressed AR, AR-V7, and HER2	Significant tumor growth inhibition without weight loss (22Rv1)
3ClQuin-SAHA, 3BrQuin-SAHA[84]	Hydroxamic acid, EGFR-targeting gefitinib-type anilinoquinazoline cap	Higher antiproliferative activity than gefitinib (DU145 cells), HDAC inhibition, EGFR inhibition, mTOR suppression	-
CUDC-907 (fimepino-stat)[86]	Hydroxamic acid, PI3K-targeting thienopyrimidine cap	High growth inhibitory activity, inhibition of HDACs and PI3K, apoptosis induction, increased Bim, suppressed Mcl-1 and Bcl-xL, suppressed DNA repair and DNA damage response proteins (Wee1, CHK1, RRM1, and RRM2), suppressed *c*-Myc	Tumor growth inhibition by 60% without weight loss (LuCaP 35CR patient-derived mouse xenografts)

AR: Androgen receptor; EGFR: epidermal growth factor receptor; HER2: human epidermal growth factor receptor 2; mTOR: mammalian target of rapamycin; PI3K: phosphatidylinositol 3-kinase.

The adamantyl-capped HDAC inhibitor CN133 was superior to vorinostat in terms of Class I HDAC1, -2, and -3 inhibition (IC_{50} = 0.6, 2, and 0.3 nM for CN133; 4, 11, and 3 nM for vorinostat), while vorinostat was more active against HDAC6 (IC_{50} = 2 nM) than CN133 (IC_{50} = 4.1 nM). CN133 was 100 times higher antiproliferative (IC_{50} = 10 nM) in 22Rv1 CRPC cells than vorinostat (IC_{50} = 1 μM). CN133 inhibited CRPC cell migration and invasion and suppressed AR signaling. Mice bearing 22Rv1 CRPC were treated with CN133 (1 mg/kg), which reduced tumor volume and tumor weight by 50% in comparison with placebo group mice[71].

Hybrid molecules targeting two or more anticancer targets can possess higher anticancer activities accompanied by reduced drug-drug interactions and less complex pharmacokinetics[72,73]. Several chimeric HDAC inhibitors with dual or multimodal activities were reported over the last years[29,30]. Compound 2-75 is a promising enzalutamide hybrid with HDAC inhibitory activity, which induced p21, led to higher acetyl-tubulin levels (based on stronger HDAC6 inhibition) than vorinostat, and suppressed Hsp90 and AR protein levels in C4-2 prostate cancer cells[74]. Based on these results, deeper studies of 2-75 in CRPC were carried out. Compound 2-75 suppressed DHT-induced AR transcriptional activity and AR translocation to the nucleus stronger than enzalutamide. In addition to AR, the mutant AR-V7 was also downregulated by 2-75 in prostate cancer cells in a proteasome-dependent way, indicating enhanced AR degradation in 2-75-treated cells. *In vivo* experiments with LNCaP tumor models revealed that 2-75 treatment (10 mg/kg, intratumoral injection twice weekly) had tumor growth inhibitory activity similar to enzalutamide, but, in the long run (after Day 24), 2-75 displayed improved tumor growth inhibition when compared with enzalutamide. The *in vivo* activity of 2-75 was accompanied by increased apoptosis induction and suppressed AR nuclear accumulation in the tumor bodies of treated mice[75].

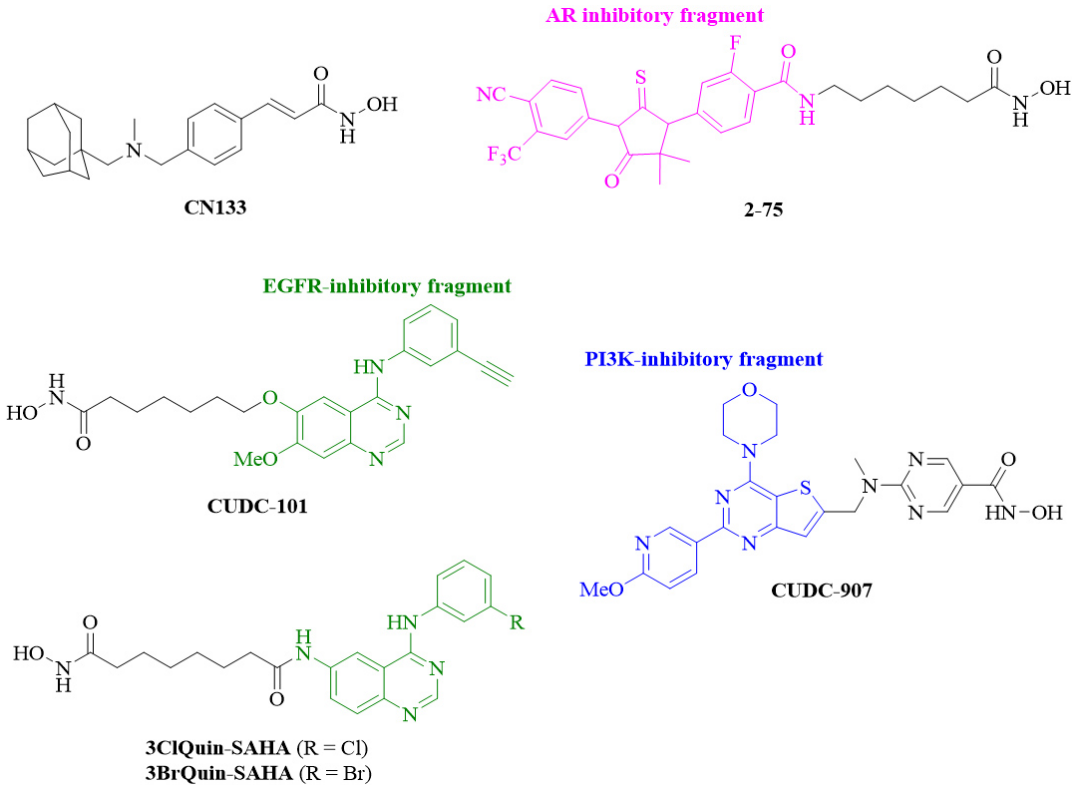

Figure 4. Structures of new HDAC inhibitors with promising activities against CRPC. HDAC: Histone deacetylase; CRPC: castration-resistant prostate cancer; AR: androgen receptor; EGFR: epidermal growth factor receptor; PI3K: phosphatidylinositol 3-kinase.

The aforementioned receptor tyrosine kinases such as EGFR are excellent targets for the design of new HDAC/EGFR inhibitors. Compound CUDC-101 combines an HDAC inhibitory fragment with an EGFR inhibitory scaffold derived from the approved anticancer active EGFR inhibitor erlotinib and inhibited HDAC, EGFR, and HER2[76]. In CRPC cells, CUDC-101 suppressed full-length AR as well as the variant AR form AR-V7, upregulated p21, and downregulated HER2/NEU. In castrated mice bearing aggressive 22Rv1 CRPC tumors, CUDC-101 (50 μg/kg/day for 14 days) inhibited tumor growth significantly without measurable weight loss of treated mice[77].

Erlotinib and CUDC-101 have certain drawbacks, which warranted further research efforts. The ethinylphenyl residue of erlotinib can be activated by cytochrome P450 enzymes leading to oxidized phenol and quinone compounds with certain toxicity potential[78]. Indeed, erlotinib was reported to increase the risk of lethal gastrointestinal tract perforations in cancer patients taking corticosteroids or ciprofloxacin[79]. A clinical phase 1 study of CUDC-101 together with chemoradiation in head and neck squamous cell carcinoma (HNSCC) patients reported that five out of twelve HNSCC patients (i.e., 41%) had to stop the therapy due to adverse events[80]. In addition, CUDC-101 was shown to be a substrate of the cell-detoxifying ABC transporters ABCB1 (P-gp) and ABCG2 (BCRP) leading to resistance to CUDC-101 treatment, whereupon the combination with P-gp and/or BCRP inhibitors was suggested in order to avoid CUDC-101 resistance formation[81]. In contrast to that, the approved EGFR inhibitor gefitinib reversed P-gp- and BCRP-mediated drug resistance *in vitro* and *in vivo*[82,83]. Hence, gefitinib is a promising conjugation partner, and chimeric HDAC inhibitors with gefitinib-derived cap scaffolds based on halogen-substituted aniline rings were prepared. The chimeric compounds 3ClQuin-SAHA and 3BrQuin-SAHA showed 3-4 times higher growth inhibitory activity (IC_{50} = 3.23 μM for 3ClQuin-SAHA and 3.53 μM for 3BrQuin-SAHA)

against DU145 CRPC cells than gefitinib (IC_{50} = 11.9 μM); however, vorinostat (IC_{50} = 0.68 μM) was still more antiproliferative in these prostate cancer cells. Nevertheless, 3ClQuin-SAHA and 3BrQuin-SAHA combined EGFR inhibitory activity with HDAC inhibition, suppressed EGFR expression comparable to vorinostat, showed only marginal unspecific toxicities, induced apoptosis in DU145 cells, and inhibited angiogenesis[84]. Hence, these chimeric compounds can be suitable anticancer drug candidates in prostate cancer therapy in terms of reduced erlotinib (and vorinostat) toxicity and resistance formation.

CUDC-907 (fimepinostat) is another promising HDAC/kinase inhibitor, which was designed to target HDAC enzymes and the kinase PI3K[85]. Based on previous reports describing the synergistic effects of CUDC-907 in various cancer models and since PI3K signaling also plays an eminent role for CRPC development (see the aforementioned role of mTOR inhibitors), CUDC-907 was investigated in prostate cancer models. CUDC-907 showed excellent growth inhibitory activities in a panel of eight prostate cancer cell lines with IC_{50} values between 2 and 17.4 nM, inhibited HDACs and PI3K signaling, and induced apoptosis in a dose-dependent way associated with increased pro-apoptotic Bim and suppressed anti-apoptotic Mcl-1 and Bcl-xL expression in 22Rv1 CRPC cells. In addition, CUDC-907 treatment led to enhanced DNA damage due to downregulated DNA damage response proteins (Wee1, CHK1, RRM1, and RRM2). The protein expression of the oncoprotein *c*-Myc, which regulates the mentioned apoptosis factors and DNA damage response proteins, was also suppressed by CUDC-907 and, thus, *c*-Myc suppression plays a key role in the anti-prostate cancer mode of action of CUDC-907. Finally, CUDC-907 (100 mg/kg/day, p.o.) was tested in castration-resistant LuCaP 35CR mouse xenografts and inhibited *in vivo* tumor growth by ca. 60% while no weight loss was detected[86]. More recently, a phase 2 study of oral CUDC-907 for the treatment of relapsed/refractory diffuse large and high-grade B-cell lymphoma (DLBCL and HGBL) patients with enhanced Myc-expression and/or altered *MYC* gene constitutions (e.g., altered gene composition by translocation) was published revealing an overall response rate of 22% in the MYC-altered disease patients[87]. Hence, patients enrolled for future prostate cancer clinical studies with oral CUDC-907 should be tested for their MYC-status. In contrast to CUDC-101, CUDC-907 was not a substrate of P-gp (ABCB1) transporters, and its antiproliferative activity was conserved in P-gp-expressing tumor cells. However, CUDC-907 was still efficiently inactivated by BRCP (ABCG2) transporters, similar to CUDC-101, and combinations with BCRP inhibitors were recommended to keep the high anticancer activity of CUDC-907[88].

CONCLUSION

HDAC inhibitors exhibit reasonable *in vitro* and *in vivo* activities against CRPC. Clinical trials have shown that monotherapy with HDAC inhibitors cannot be recommended now for phase 3 studies due to low efficacy and considerable toxicity in CRPC patients. However, the combination of HDAC inhibitors with certain anticancer drugs such as taxanes and anti-androgens appeared promising based on the data from clinical studies. In preclinical studies, DNA methylation inhibitors, PARP inhibitors, various protein kinase and mTOR inhibitors, interferon, and TRAIL gene therapy appeared to be suitable combination partners for HDAC inhibitors, which should be considered for future clinical studies with CRPC patients.

Various new HDAC inhibitors have emerged over the last years, which displayed significant activity against CRPC in preclinical studies. Many of these new HDAC inhibitors were designed to target non-HDAC proteins such as protein kinases or the AR in addition to HDAC enzymes. Among them, CUDC-101 and CUDC-907, which are HDAC/kinase inhibitors, are striking examples that have already entered clinical trials. The activities of these two drug candidates were thoroughly studied in preclinical CRPC models giving hints at strengths and weaknesses of multi-targeting HDAC inhibitors. Strong CRPC growth inhibition accompanied by good drug tolerance in mice were contrasted by toxicity in patients and

resistance formation based on the expression of ABC transporters in tumor cells. Efforts to eliminate the drawbacks of CUDC-101 and CUDC-907 are already in progress and the first preclinical results of new HDAC/kinase inhibitory derivatives are available. In addition, subgroups of CRPC patients with increased Myc levels should be considered for future clinical trials with CUDC-907 since lymphoma patients with elevated Myc responded well to this sophisticated HDAC inhibitor.

The drug developments described in this work are based on the elucidation of the mechanisms of action of first-generation HDAC inhibitors, the design of new therapy regimens, and the synthesis of new HDAC inhibitors with improved anticancer properties. The close interplay of clinicians, tumor biologists, and synthetic chemists has led to promising outcomes in the field of CRPC research over the last years, and interdisciplinary work will continue to help HDAC inhibitor-based therapy to play a prominent role as efficient and well-tolerated CRPC treatment in the future.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study, provided administrative and technical support: Nitzsche B, Höpfner M

Performed data acquisition, data analysis and interpretation: Biersack B

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All authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

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Approaches to identifying drug resistance mechanisms to clinically relevant treatments in childhood rhabdomyosarcoma

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Abstract

Aim: Despite aggressive multiagent protocols, patients with metastatic rhabdomyosarcoma (RMS) have poor prognosis. In a recent high-risk trial (ARST0431), 25% of patients failed within the first year, while on therapy and 80% had tumor progression within 24 months. However, the mechanisms for tumor resistance are essentially unknown. Here we explore the use of preclinical models to develop resistance to complex chemotherapy regimens used in ARST0431.

Methods: A Single Mouse Testing (SMT) protocol was used to evaluate the sensitivity of 34 RMS xenograft models to one cycle of vincristine, actinomycin D, cyclophosphamide (VAC) treatment. Tumor response was determined by caliper measurement, and tumor regression and event-free survival (EFS) were used as endpoints for evaluation. Treated tumors at regrowth were transplanted into recipient mice, and the treatment was repeated until tumors progressed during the treatment period (i.e., became resistant). At transplant, tumor tissue was stored for biochemical and omics analysis.

Results: The sensitivity to VAC of 34 RMS models was determined. EFS varied from 3 weeks to > 20 weeks. Tumor models were classified as having intrinsic resistance, intermediate sensitivity, or high sensitivity to VAC therapy. Resistance to VAC was developed in multiple models after 2-5 cycles of therapy; however, there were examples where sensitivity remained unchanged after 3 cycles of treatment.

Conclusion: The SMT approach allows for *in vivo* assessment of drug sensitivity and development of drug resistance in a large number of RMS models. As such, it provides a platform for assessing *in vivo* drug resistance mechanisms at a "population" level, simulating conditions *in vivo* that lead to clinical resistance. These VAC-resistant models represent "high-risk" tumors that mimic a preclinical phase 2 population and will be valuable for identifying novel agents active against VAC-resistant disease.

Keywords: Rhabdomyosarcoma, patient-derived xenografts, combination therapy, intrinsic drug resistance, acquired drug resistance

INTRODUCTION

Advanced or metastatic rhabdomyosarcoma (RMS) has a poor prognosis. Patients with stage 4 disease, with the exception of those with embryonal RMS that are less than ten years of age, have a long-term event-free survival (EFS) of less than 20%[1-3]. Current therapies include all the known active agents plus radiation and surgery, but neither intensification of chemotherapy by increasing the dose of cyclophosphamide and adding active agents to standard VAC therapy (Vincristine/Actinomycin-D/Cyclophosphamide), nor use of high-dose chemotherapy with stem-cell rescue has improved outcome over the last 30 years[4]. The failure of current therapeutic approaches to effectively treat advanced or metastatic RMS despite numerous cooperative group trials, is a consequence of intrinsic or acquired resistance to the limited number of drugs that compose the armamentarium for treating this disease. Even with the use of intensive multimodality treatment, only 20% of patients will be long-term survivors. The failure of current therapies is further emphasized by the rapid progression rate of patients enrolled in a recent high-risk rhabdomyosarcoma study (ARST0431). In this trial, 25% of patients diagnosed with metastatic disease, either failed during the 52 weeks of treatment or had an event within two years (80%)[5]. The causes of tumor resistance are poorly understood. One possible cause for resistance is tumor clonal evolution, where novel mutations acquired during therapy confer resistance[6]; however, such studies usually rely on small cohorts as relatively few tumors are re-biopsied at relapse.

Preclinical studies aimed at understanding resistance tend to focus on individual drugs, rather than resistance to poly-chemotherapy[7-13]. Further, often drug exposures used *in vitro* far exceed those achieved in patients, and escalating drug concentrations over time do not simulate how clinical resistance is acquired[14]. We have previously used pediatric tumor xenografts to select for acquired resistance in mice[7,8]. However, while this approach is perhaps more appropriate than *in vitro* selection, conventional testing is resource-intensive, and allows for only a few models to be explored[15]. More recently, we have adopted Single Mouse Testing (SMT) to evaluate drugs in a large number of xenograft models[16,17], an approach that significantly increases the inclusion of genetic diversity for a given cancer type[18]. The SMT design is validated by a retrospective analysis of > 2100 tumor-drug studies undertaken by the Pediatric Preclinical Testing Program, where the response of a tumor in one mouse, selected at random from the group, was compared to the median group response. This analysis showed that the SMT accurately predicted responses in 78% of studies. Allowing for a deviation of ± one response classification [e.g., stable disease (SD) *vs.* partial response (PR)], the concordance was 95%. Further, the SMT analysis was accurate in identifying the antitumor activity of 66 of 67 drugs in terms of the objective response rate determined for each drug over a range of tumor models[18]. Prospective studies with up to 90 ALL models and up to 50 solid tumor models

show that SMT has similar concordance with conventional testing[16]. Importantly, using SMT, one can potentially incorporate up to 20-fold the number of models for evaluation of an agent, encompassing many diseases, or encompassing the genetic diversity of a given disease. Here we have applied SMT to interrogate intrinsic resistance and to use this approach to select for acquired resistance *in vivo* to VAC therapy in 34 RMS models. Our data show that RMS models have a range of sensitivities to VAC, ranging from tumor progression through initial treatment to maintained complete response at week 20 after a single cycle of VAC treatment. Further, using the SMT design, acquired resistance can be developed over a period of 3 to 5 cycles of therapy.

METHODS

C.B.17SC *scid*$^{-/-}$ (C.B-*Igh*-1^{b}/IcrTac-*Prkdc*^{scid}) female mice (Envigo, Indianapolis, IN) were maintained under barrier conditions, and experiments were conducted using protocols and conditions approved by the Institutional Animal Care and Use Committee at UTHSA as previously described[15]. Mice were selected for VAC treatment when tumors were 200-300 mm^3. Regrowth of tumors was determined following tumor regression. Endpoints were EFS, defined as tumor growing to 400% of its volume at the initiation of treatment, and percent tumor volume regression. Complete regression (CR) was defined as tumor volume < 40 mm^3 (the level of detection). Limited demographic data for RMS models used in the studies are presented in Table 1. Patient- and cell-derived xenograft models (PDX and CDX) have been described previously[15,17]. Genomic data for some of the models is under https://ocg.cancer.gov/programs/target/ Pan-cancer Model Systems, and in Rokita *et al.*[19]. SJRHB011_X, SJRHB013_X, SJRHB010927_X1, SJRHB000026_X1, and SJRHB013758_X1 were obtained from The Childhood Solid Tumor Network[20]. SMS-CTR cells were obtained from Dr. Corine Linardic, Duke University, JR1 (UK), and CCA cells were obtained from Dr.Marielle Yohe, NCI. All tumors were authenticated by short tandem repeat analysis against reference profiles.

Simulating VAC therapy in mice

All drugs were administered IP. Vincristine was administered at 0.5 mg/kg days 1,8,15, actinomycin D (0.20 mg/kg) and cyclophosphamide (120 mg/kg), both on day 1, doses considered to give clinically relevant drug exposures[21-23]. All drugs were formulated in 0.9% w/v saline. This regimen, which simulates VAC modules used in ARST0431, was tolerated with ~10% body weight loss, and mortality < 2%.

Development of acquired resistance in mice

The schema for selecting for acquired resistance is presented in Figure 1. Briefly, one mouse per tumor line received a single cycle of VAC treatment. Tumor diameters were measured weekly, and treated tumors were re-transplanted into new recipient mice when they "evented" (achieved 400% of their volume on day 1 of treatment). Treated tumors were considered resistant to VAC therapy if they progressed through treatment and had increased > 25% in volume by day 21. Tumor tissue was cryopreserved and snap frozen after each cycle of VAC therapy.

RESULTS

Intrinsic sensitivity to VAC treatment

We initially explored the sensitivity of 34 RMS xenograft models to the "gold standard" therapy for RMS, namely VAC, Figure 2. The models encompass both fusion-positive (alveolar), and fusion-negative (embryonal, with and without RAS mutations). As shown in Figure 2A, 18 of 34 xenograft models (53%) regressed > 50% on Cycle 1 of VAC therapy, the other models showing SD (n = 3) or progressive disease (PD) (n = 13). This experiment is ongoing, but clearly shows that SMT can be valuable for assessing drug sensitivity in a large cohort of models. Thirteen models were intrinsically resistant to VAC treatment with EFS of < 40 days, whereas others (n = 10) are highly sensitive (EFS > 70 days), with four models remaining

Table 1. Characteristics of RMS xenograft models and response to cycle 1 of VAC therapy

Model ID	Subclass	Age (years)	Gender	Site	Diagnosis or relapse	Response	EFS (weeks)
Rh10	ARMS	15	Female	Liver	Relapse	PD	6
Rh28	ARMS	17	Male	Hand	Diagnosis	MCR	> 20
Rh30R	ARMS	16	Male	Bone marrow	Relapse	CR	9
Rh41	ARMS	12	Female	Unknown	Autopsy	PD	3
Rh18	ERMS	2	Female	Perineum	Diagnosis	CR	10
Rh36	ERMS	15	Male	Paratesticular	Relapse	PR	6
Rh66	ARMS	12	Female	Axillary lymph node	Metastasis	CR	7
Rh12	ERMS	12	Male	Right buttock	Diagnosis	MCR	> 20
NCH-ERMS-1	ERMS	5	Male	orbital	No treatment	PD	5
NCH-ARMS-2	ARMS, metastatic	16	Female	Right breast	Treated	PD	4
Rh71	ERMS, high grade	17	Male	Prostate	Diagnosis	PD	6
Rh72	ERMS	3	Female	Perineal	Treated	PD	9
Rh73	ERMS	5	Male	Right infratemporal fossa mass	Treated	PD	7
Rh75	ERMS	17	Male	Pelvis recurrent (Rh71)	Treated	CR	8
Rh78	ARMS	1	Male	Right thigh	No treatment	SD	8
Rh80	ERMS anaplastic	5	Female	Stomach mass	Treated	PD	5
Rh81	ERMS	9	Male	Abdominal mass	Treated	PR	7
Rh82	ARMS	3	Male	Paratesticular	Treated	PR	10
Rh83	ERMS	4	Male	Left orbital mass	Treated	PR	14
Rh84	ARMS	2	Male	Upper lip lesion	Treated	SD	12
Rh85	ERMS diffuse anaplasia	5	Female	Abdominal mass	No treatment	PD	4
Rh86	ERMS	8	Male	Retroperitoneal mass	Treated	CR	10
Rh87	Spindle cell/sclerosing	6	Female	Oropharyngeal mass	Treated	PD	3
Rh88	ERMS	10	Male	Pelvic mass	Treated	CR	12
IRS-56	ERMS	3	Male	Buttock	Diagnosis	CR	> 20
IRS-68	ERMS	13	Male	Shoulder	Diagnosis	PR	7
SJRHB011_X	ERMS	5	Male	Infratemporal fossa	Recurrent	PD	4
SJRHB013_X	ERMS	3	Female	Perineal/bladder	Recurrent	CR	13
SJRHB010927_X1	ERMS	5	Female	Parapharyngeal	Diagnosis	PR	12
SJRHB000026-X1	ERMS	4	Female	Pelvis	Recurrent	PD	5
SJRHB013758_X1	ERMS	4	Female	Abdomen/pelvis	Diagnosis	CR	10
SMS-CTR*	ERMS	1	Male	Pelvis	Diagnosis	MCR	> 20
JR-1 (UK)*	ERMS	7	Female	Lung	Relapse	PD	7
RD*	ERMS	7	Female	Pelvis	Relapse	SD	11

*Indicates cell line derived xenograft. ARMS: Fusion-positive; ERMS: fusion negative; RMS: rhabdomyosarcoma; VAC: vincristine/actinomycin-D/cyclophosphamide; EFS: event-free survival; PD: progressive disease; MCR: maintained complete response; CR: complete regression; PR: partial response; SD: stable disease.

in CR at 120 days, as shown in the Kaplan-Meier plot [Figure 2B]. We defined PD as tumors that increased in volume by 25% at day 21 (6 days after the last dose of vincristine). Thirteen of 34 RMS models (38%) exhibited PD to cycle 1 of VAC. An EFS of 49 days or less was also observed for 13 models, Table 1. Of these 13 intrinsically resistant models, 10 tumors were established from patients following treatment. The exceptions were Rh71, Rh85, and NCH-ERMS1, which were established as PDX models from patients prior to any treatment. Other models with short EFS (CCA, Rh36, IRS-68) each regressed > 50% (i.e., PR), but

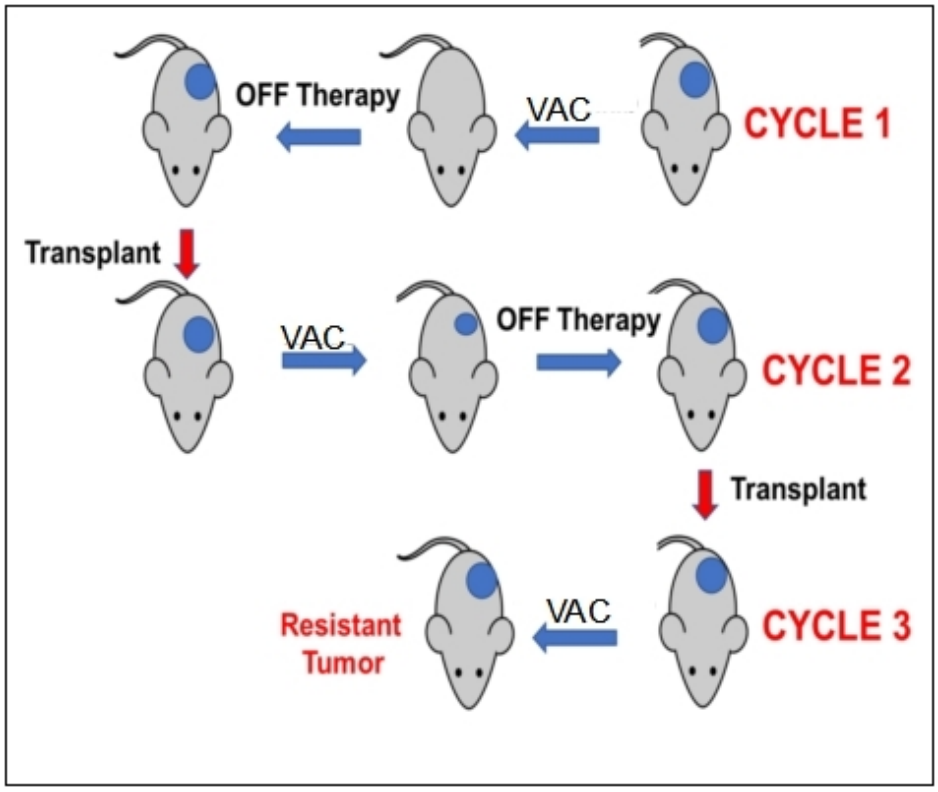

Figure 1. Schema for developing resistance to VAC therapy in mice using the single mouse study design. Mice received a single cycle of VAC (vincristine, actinomycin D, cyclophosphamide). Tumor response was determined, and tumors were transplanted in recipient mice when they achieved 400% of their volume on the first day of treatment.

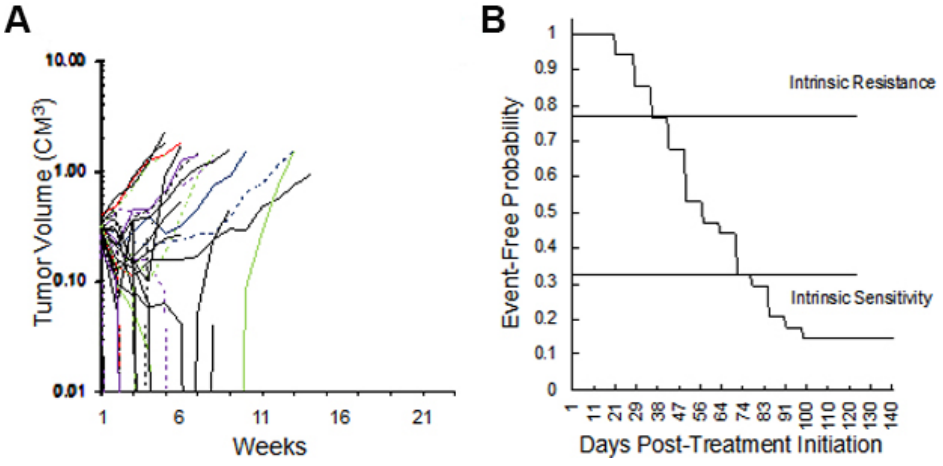

Figure 2. (A) Initial volume responses of 34 RMS models to cycle 1 of vincristine + actinomycin D + cyclophosphamide (VAC) using the SMT experimental design (ongoing expt.). (B) Kaplan-Meier plot for EFS of the same models. RMS: Rhabdomyosarcoma; VAC: vincristine/actinomycin-D/cyclophosphamide; EFS: event-free survival; SMT: single mouse testing.

rapidly regrew. Of the models having EFS of 70 days or greater (n = 10; 29%), 3 models had CR (Rh88, SMS-CTR, SJRHB013x, and SJRHB013758_X1), 2 had PR (SJRHB010927, Rh83), and 1 had prolonged SD (Rh84). Three models, established from tumor at diagnosis, were highly sensitive to VAC treatment with no evidence of tumor regrowth at day 120 after the start of treatment (IRS-56, Rh12, Rh28). The other 11

models had intermediate sensitivity to VAC (EFS > 40 but < 70 days).

Development of acquired resistance

The approach to selecting for acquired resistance [Figure 1], was chosen as there is an increased incidence of scid lymphoma as mice age, and also potentially altered immunity that could alter the response to chemotherapy. Consequently, we re-transplanted tumors at 400% of their volume at day 1 of treatment into new 6 to 8-week-old female C.B.17SC *scid*$^{-/-}$ mice, and repeated this procedure until tumors became resistant. For all studies, tumors were allowed to grow to 200-300 mm^3 before starting treatment. Initially, we transplanted cycle 1 tumor into two mice, in case there was toxicity, and we would lose the model if there was a death (drug related or unrelated) on cycle 2 of treatment. However, there was no mortality, so subsequently, a single mouse design was used for all subsequent cycles of VAC treatment. Where two mice were used (n = 16), the responses between tumors were very similar, Supplemental Figure 1. The "Swimmer plot" in Figure 3 shows examples of models exhibiting Complete Regression (Rh88) or Partial Regression (IRS-6) to VAC treatment on cycle 1 with rapid emergence of resistance, or tumor models that initially had progressive disease (i.e., intrinsically resistant), but where EFS shortened with further cycles of VAC treatment (Rh72), or a model where EFS remained constant during 3 cycles of VAC treatment (Rh73).

DISCUSSION

The outcome for patients with high-risk RMS has not improved significantly for several decades. In part, this is because new effective agents have not been identified, and agents such as irinotecan, while active against RMS[24,25], do not increase overall survival when added to VAC therapy[5,26]. Of the chemotherapeutic agents used in high-risk protocols such as ARST0431, all but one (vincristine), induces DNA damage. However, the mechanism(s) that confer resistance to multi-chemotherapy protocols, even protocols that combine mostly DNA damaging agents, remains unknown. A proportion of patients failing on therapies including cyclophosphamide and vincristine[27], however, may respond to vinorelbine/cyclophosphamide[28].

Approaches to determine mechanisms of resistance, both *in vitro* and *in vivo*, have limitations. As mentioned previously, *in vitro* studies may not accurately replicate clinical drug exposures, and continuous exposure to increasing drug levels may result in multidrug resistance mechanisms, such as overexpression of ABC transporters, that may not reflect clinical reality[29]. In vivo studies in mice, using human tumor xenografts, or syngeneic tumor models, may more accurately model drug pharmacokinetics relevant to clinical exposures. However, resource constraints tend to limit their use to a few models and usually for the development of resistance to single agents. One approach to encompassing genetic diversity for a given cancer type is to use an SMT design, rather than conventional experimental designs that use 8-10 mice for control and treatment groups[15,18]. In this pilot study, we have evaluated SMT in the context of VAC therapy in 34 RMS models, and have used this approach to develop models with acquired resistance that can be further characterized by "omics" and biochemical methods. Essentially, the approach mimics a clinical trial in that there is no "control" arm, and the response criteria are tumor volume changes and EFS. We "enrolled" RMS models when tumors were available, and the response to one cycle of VAC was assessed in a blinded manner with respect to whether the model was established from an untreated patient or from a patient following treatment. As VAC therapy has been the backbone for the treatment of RMS for over 30 years, we assume that xenografts established from treated patients received these drugs, although it is not known if additional drugs were administered to patients before their tumors were established as xenografts.

Overall the models appear to retain drug sensitivity characteristics of patient tumors. Ten of the 13 models that had PD to VAC treatment were derived from pre-treated tumors (treated, relapse, autopsy), whereas only 2 out of 11 models established from diagnosis samples progressed on cycle 1 of VAC treatment. The

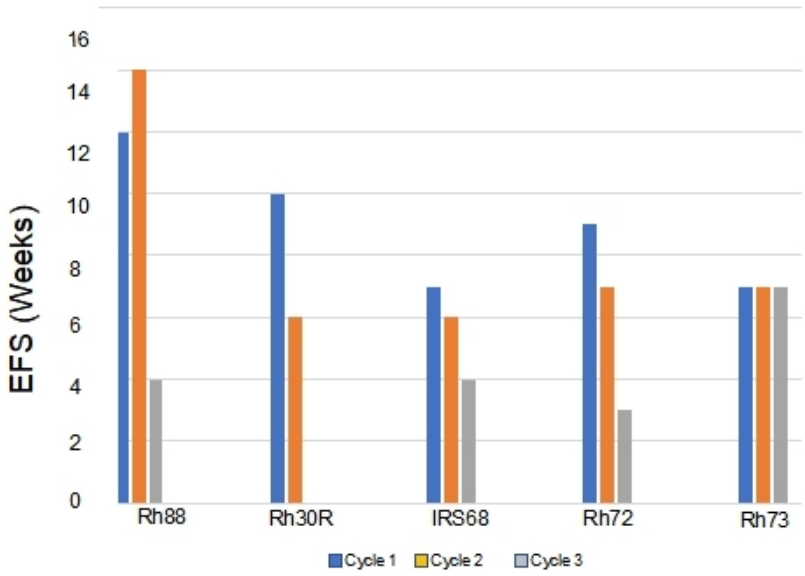

Figure 3. Development of resistance to VAC treatment (decreases in EFS) in models that were intrinsically sensitive to treatment (> PR; Rh88, Rh30R, IRS-68) or models where tumors progressed through cycle 1 of VAC treatment (Rh72, Rh73). VAC: Vincristine/actinomycin-D/cyclophosphamide; EFS: event-free survival; PR: partial response.

models established as PDX's from samples at diagnosis that were intrinsically resistant to VAC included two ERMS, one defined pathologically as a high-grade tumor (Rh71) and one with diffuse anaplasia (Rh85), which may relate to drug sensitivity[30,31]. Tumor models having EFS of 70 days or greater included 7 established from diagnosis samples, 5 listed as "treated", and 3 models established from tumor at relapse. Clinically, patients progressing on front-line treatment may respond to phase 2 protocols, including cyclophosphamide and *vinca* alkaloids. It is also possible that prolonged passage of tumor in untreated mice could lead to loss of resistance, or overgrowth of sensitive cells. However, the data set from 34 models serves as a reference point for understanding intrinsic resistance or sensitivity and for developing acquired resistance in those models that initially respond to VAC treatment under relevant in vivo conditions.

We have used two criteria for assessing tumor sensitivity to VAC: tumor regression (partial and complete response) and EFS. Tumors having PD had EFS times of 3-8 weeks; those demonstrating PR (> 50% volume regression) had EFS that ranged from 6-14 weeks, and models having CR the EFS time ranged from 7 to > 20 weeks. Thus, as in our previous study[17], there was a relatively poor correlation between the magnitude of tumor volume regression and EFS, suggesting that EFS may be a more reliable metric of tumor response in preclinical models. Clinical data also question the value of early tumor response as a prognostic indicator, as neither computed tomography and/or magnetic resonance imaging predicted outcomes for patients with rhabdomyosarcoma[32,33].

We have attempted to select for acquired resistance to VAC using the SMT approach. While this pilot study is far from complete, interim analysis suggests that this approach can generate a large number of VAC-resistant models that can be analyzed for genetic/epigenetic changes associated with resistance. Thirteen xenograft models showed progressive disease in response to VAC treatment, hence were classified as intrinsically resistant. However, many of these resistant tumors had reduced EFS on subsequent cycles of therapy, thus showing increased resistance. In ongoing studies, acquired resistance has been selected within 3 to 5 cycles of VAC treatment in many tumor models initially sensitive to VAC. The approach we have taken is to define intrinsic sensitivity/resistance to VAC and to select for acquired resistance in mice. An

alternative approach is to establish PDX models at diagnosis and relapse from the same patient. This is rarely possible in the context of pediatric RMS, as relapse tumor is rarely biopsied, but also engraftment into mice may select for subclones rather than represent the clonal spectrum in the patient tumor. Modeling intrinsic and acquired resistance in preclinical models has an advantage over clinical studies in that changes in gene expression can be assessed both in parental and resistant tumor, but also in response to therapy[34].

Here we have defined the intrinsic sensitivity of 34 RMS models. Importantly, models intrinsically resistant to VAC, or those with acquired resistance to VAC treatment, may be particularly valuable for identifying novel agents that may be active in disease at relapse, and may have novel mechanisms of action independent of DNA damage.

DECLARATIONS

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Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Ghilu S, Morton CL, Vaseva AV, Zheng S, Kurmasheva RT, Houghton PJ

Performed data acquisition, as well as provided administrative, technical, and material support: Ghilu S, Morton CL, Houghton PJ

Availability of data and materials

All models are available to academic centers under an institutional MTA.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Experiments were conducted using protocols and conditions approved by the Institutional Animal Care and Use Committee at UTHSA. Use of patient-derived tissue was considered non-human research by the IRB.

Consent for publication

Use of patient-derived tissue was considered non-human research by the IRB. All xenografts are de-identified.

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Cancer Drug Resistance

Commentary

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Understanding sarcoma drug resistance one cell at a time

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As true for any cancer, intrinsic and acquired drug resistance represent perhaps the single biggest obstacle limiting our ability to cure patients with metastatic disease. Yet, tackling this challenge has been particularly difficult for sarcoma. First and foremost, sarcomas are rare and diverse, which splits a class of tumors representing less than 1 percent of all malignancies into more than fifty smaller molecularly and histologically distinct entities, each having its own etiology, prognosis, and clinical presentation. Even the most "common" sarcoma subtypes (e.g., liposarcoma, leiomyosarcoma, or gastrointestinal stromal tumors) have annual incidences in the thousands in the United States, whereas rarer subtypes like desmoplastic small round cell tumors number less than fifty per year. Due to their scarcity, it's not uncommon for just a few cell lines, organoids, or patient-derived tumor explants to exist for each sarcoma subtype. This shortage limits their usefulness to model the wide variety of innate and adaptive mechanisms that exist in human tumors. Many times, no representative preclinical model exists. The scarcity of FDA-approved drugs aimed at sarcoma-specific targets, often fusion proteins and transcription factors, is also problematic.

Complicating the ability to understand tumor drug resistance, many sarcomas exhibit pronounced intra-tumoral heterogeneity, manifested by heterogeneous cell populations of varied lineages and states of differentiation. It is not unusual for osteosarcoma, for example, to harbor cells with varying degrees of

chaotic osteogenic, adipogenic, or chondrogenic cell fates. Similarly, some liposarcomas are classified into well-differentiated and dedifferentiated variants by their predominant differentiation status but usually harbor a blend of both[1]. Even tumors like Ewing sarcoma, which appear relatively homogeneous by light microscopy, can be subcategorized by the pathognomonic genomic translocation expressed and by the presence or absence of well-described point mutations in *STAG2*, *p53*, or *CDKN2A*[2]. Thus, while light microscopy and careful review by an experienced sarcoma pathologist remain the cornerstone of sarcoma diagnosis, a clear trend in the research setting is to deeply interrogate each tumor and precisely catalog its cellular constituencies using a wide array of genomic, proteomic, and other "-omic" technologies. As a direct result of this unparalleled opportunity to parse out distinct groups of malignant cells, a more nuanced view of sarcoma drug resistance must consider not only the global factors linked to each sarcoma subtype but also the intratumoral features such as physical and mechanical cues (e.g., extracellular matrix, stiffness or confined adhesiveness) that allow for clonal evolution in response to the selective pressures triggered by standard chemotherapy or biologically targeted therapy.

This has been achieved in other cancer types by engaging powerful single-cell technologies - before and after drug exposure - to determine a tumor's baseline transcriptomic state and nascent cellular changes associated with drug resistance[3-6]. Single-cell and single-nucleus RNA-sequencing (sc/snRNA-seq) are the most common methods used currently and, when paired with freely available custom-made bioinformatic tools written in R or Python, provide the revolutionary ability to deeply interrogate intra-tumoral complexity[7-9]. Furthermore, while still in their infancy, single-cell proteomic technologies have introduced promising technologies that enable high-resolution spatial image - omic (SIO) analyses of human primary tumors, which allows one to learn not only *what* cell types exist but also *how* they interact in 2D or 3D space[10]. Though the clinical value of checkpoint blockade and cellular immunotherapies remains to be proven across the range of sarcoma subtypes, the advent of SIO, image mass cytometry, multi-color fluorescent microscopy, and other techniques should provide a fresh opportunity to examine whether such therapies can invoke an anti-cancer response. Complementing the trained eye of an experienced sarcoma pathologist, deep-learning and artificial intelligence, while still under-utilized, are primed to aid pathologists to make sense of the torrent of data generated from each slide and may allow them to perceive subtle cell-cell interactions that contribute to drug resistance and the feedback mechanisms that sustain them[11].

Though classic measures of drug efficacy that rely on tumor size or patient survival will persist as important clinical study endpoints, a deeper appreciation of drug sensitivity/resistance must inevitably decipher how each patient's tumor - and the cells within them - evade therapy. Like no time in history, the tools exist today to help cure cancer one cell at a time.

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Authors' contributions

Contributed to the written content and review of this manuscript: Cherradi-Lamhamedi SE, Truong D, Ludwig JA

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Cancer Drug Resistance

Review

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Tousled-like kinase 1: a novel factor with multifaceted role in mCRPC progression and development of therapy resistance

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Abstract

Standard treatment for advanced Prostate Cancer (PCa) consists of androgen deprivation therapy (ADT), but ultimately fails, resulting in the incurable phase of the disease: metastatic castration-resistant prostate cancer (mCRPC). Targeting PCa cells before their progression to mCRPC would greatly improve the outcome, if strategies could be devised selectively targeting androgen receptor (AR)-dependent and/or independent compensatory pathways which promote mCRPC development. Combination therapy by targeting the DNA damage response (DDR) along with ADT has been limited by general toxicity, and a goal of clinical trials is how to target the DDR more specifically. In recent years, our lab has identified a key role for the DDR kinase, TLK1, in mediating key aspects of adaptation to ADT, first by promoting a cell cycle arrest (through the TLK1>NEK1>ATR>Chk1 kinase cascade) under the unfavorable growth conditions (androgen deprivation), and then by reprogramming the PCa cells to adapt to androgen-independent growth via the NEK1>YAP/AR>CRPC conversion. In addition, TLK1 plays a key anti-apoptotic role via the NEK1>VDAC1 regulation on the intrinsic mitochondrial apoptotic pathway when the DDR is activated. Finally, TLK1 was recently identified as having an important role in motility and metastasis via regulation of the kinases MK5/PRAK and AKT (indirectly via AKTIP).

Keywords: TLK1, NEK1, YAP1, VDAC1, DNA damage response, prostate cancer, metastatic castration-resistant prostate cancer, drug resistance in mCRPC

INTRODUCTION

Prostate cancer (PCa) is one of the most common malignancies among men in the United States. Although death from PCa has been significantly reduced during the past two decades due to the advent of first (flutamide, nilutamide, cyproterone acetate, and bicalutamide) and second-generation (abiraterone acetate, enzalutamide, apalutamide, and recently approved darolutamide) anti-androgens, recent data of modeled projections suggests that the death rate is gradually increasing[1-4]. This might partly be due to the development of increased resistance towards androgen deprivation therapy (ADT), which eventually progresses to castration-resistant phenotype during which therapeutic options become limited. PCa is mainly a disease of uncontrolled proliferation of luminal epithelial cells of the prostate gland, although in some cases, basal cells are involved. Since androgen receptor (AR) signaling regulates the growth, survival, and proliferation of prostate tumors, the majority of PCa therapies are focused on either inhibition of androgen synthesis or blockade of androgen receptor transactivation. However, the drug effect does not last long, and the tumor relapses within 18-24 months with more aggressive phenotype known as castration-resistant prostate cancer (CRPC), even in the presence of castrate level of circulatory androgen. Besides androgen ablation, radiotherapy and chemotherapy are commonly employed for the treatment of both localized PCa and CRPC. Inflicting DNA damage and enhancing apoptosis of cancer cells are the mechanistic strategy of all radiotherapeutic (e.g., external beam radiation therapy, brachytherapy, radium-223) and some chemotherapeutic (PARP inhibitors, e.g., olaparib, niraparib, talazoparib; topoisomerase inhibitors, e.g., mitoxantrone, campothecin; platinum-based therapy, e.g., satraplatin, carboplatin, cisplatin; DNA crosslinking agent, e.g., mitomycin C) interventions (reviewed in Ref.[5,6]). It is observed that AR signaling is still active in the majority of CRPC due to the amplification, mutation, overexpression, and alternative splicing of AR that directly regulates the expression of DNA repair genes[5,7-10]. Therefore, a combinatorial treatment including ADT and other DNA damage-inducing agents should result in favorable clinical outcome and increase patients' survival. Despite the improvement of treatment modalities, CRPC remains incurable, and drug-refractory PCa is evident among patients. Interestingly, other compensatory oncogenic survival mechanisms might play a role in conjunction or independent of AR signaling. Several receptor tyrosine kinases and mitogen-activated protein kinases mediated signaling are found to be activated in CRPC, which can bypass AR requirement for PCa cell growth and proliferation[11-13]. In this communication, we will focus on a less commonly reported serine/threonine kinase, TLK1 and its compensatory role in developing therapy resistance and metastatic CRPC (mCRPC) progression.

THE MAMMALIAN TOUSLED LIKE KINASES AND TRANSLATIONAL UPREGULATION OF TLK1B

Mammalian homologues of plant tousled-kinase, known as tousled-like kinases (TLKs), were first cloned in 1999 and found to be implicated in DNA replication as their activities peak during the S-phase of the cell cycle[14]. Two members of the tousled-like kinase family have been identified (TLK1 and TLK2), which were found to share similar substrates and have partly redundant functions in maintaining genomic integrity. Later on, detailed studies of these two proteins identified their individual functions in various cellular processes along with their shared ones. TLK1 is reported to directly play a role in DNA replication, transcription, cell cycle checkpoint control, DNA damage response and repair (reviewed in Ref.[15-17]). Although multiple isoforms of TLK1 are discovered in different tissues, a translationally controlled isoform TLK1B has been thoroughly characterized by our group. TLK1B is an N-terminal truncated spliced variant of TLK1, which possesses a long, GC rich 5" UTR and two short upstream open reading fames,

characteristics of a weakly competitive mRNA. TLK1B possesses an intact C-terminal catalytic domain, and its substrates specificity is similar to TLK1. A schematic sequence alignment of TLK1 and TLK1B can be found in Ref.[18]; while the alignment of TLK1 and its homolog TLK2 can be found in Ref.[16,19]. Translation of TLK1B is facilitated by the overexpression of eIF4E or by the stress-related activation of the AKT-mTOR pathway, which leads to 4EBP1 hyperphosphorylation and release of eIF4E from its inhibitor[18,20,21]. Histone H3 was one of the first identified substrates which can be phosphorylated on serine 10 residue by TLK1B. Although some other kinases (Aurora B/Ipl1 kinase) may also phosphorylate H3-S10, TLK1B phosphorylation of Histone H3-S10 promotes proper chromosomal condensation during metaphase and confers radio-resistance as observed in normal mouse breast epithelial cells (MM3MG) and yeast[22]. Another direct substrate of TLK1/1B is Histone H3-H4 chaperone Asf1a and Asf1b, which can be phosphorylated on several residues at C-terminus and increase their affinity to bind to H3-H4 that enables nucleosomal assembly with newly synthesized DNA strand during replication and damage repair[23,24]. TLK1B is also reported to phosphorylate Rad9 of Rad9-Rad1-Hus1 (9-1-1) heterotrimeric complex that acts as a scaffold to hold the DNA at the damaged region and recruits DNA repair factors[25]. Following DNA repair, TLK1/1B mediated phosphorylation of Rad9 at S328 promotes its dissociation and disassembly from the 9-1-1 trimeric complex and resumes the cell cycle from DNA damaged induced checkpoint arrest[26]. Besides these well-validated substrates of TLK1/1B, a proteomic screening from our lab identified 165 binding partners of TLK1/1B from a pool of 9000 full-length human proteins[27]. The physiological functions of the majority of these interactions are still unknown.

TLKs play a central role in DNA damage response and repair and hence, are critical for cell survival. However, studies aimed at identifying TLKs' role in prostate cancer progression and drug resistance are very limited. Notably, the *TLK1* gene was identified by co-expression analysis using WGCNA as a key driver of PCa, highly enriched among candidate genes collected from expression Quantitative Trait Loci, somatic copy number alterations and prognostic analyses[28]. We also recently observed from data mining that TLK1 expression at mRNA level is increased in metastatic and high grade prostatic adenocarcinoma compared to the low risk tumors[29]. Moreover, androgen deprivation translationally increases TLK1B level in LNCaP cells and a PDX mice model through an mTORC1 dependent mechanism without increasing TLK1 mRNA expression; and mTOR inhibition by rapamycin reverses this phenomenon[30,31]. This compensatory activation of mTOR during AR blockade might be due to the oncogenic activation of AKT. AR inhibition prevents PHLPP mediated dephosphorylation of AKT by downregulating the expression of FKBP5[32,33]. The immunophilin *FKBP5* (FK506-binding protein 5) is an androgen-responsive gene whose scaffolding activity is required for PHLPP to dephosphorylate AKT at S473, a site on which phosphorylation is necessary for full activation of AKT[34,35]. In addition, FKBP5 enables the pharmacologic activity of rapamycin by binding to it that allows the association of FKBP5-rapamycin complex to the FKBP5-rapamycin binding domain (FRB domain) of mTORC1. FKBP5-rapamycin binding to FRB directly inhibits mTORC1 by masking its substrates docking sites or dissociating the regulatory subunits from the mTORC1 complex (reviewed in Ref.[36]).

ROLE OF TLK1 IN PCA PROGRESSION AND DRUG RESISTANCE

The functional implications of TLK1 overexpression and/or TLK1B translational upregulation have multi-faceted role in driving drug resistance and hence, promoting PCa aggressiveness. Our group first demonstrated that TLK1/1B can mediate DNA damage response (DDR) through NIMA-related kinase 1 (NEK1). TLK1/1B tightly binds and phosphorylates NEK1 both *in vitro* and *in vivo* and this association is strengthened during DNA damage events[27]. Several studies reported NEK1 as a crucial factor in mediating the DDR along with its functions in other cellular processes, like the regulation of mitosis. In response to genotoxic threats, NEK1 is translocated to DNA damage foci in the nucleus and triggers the cell cycle

checkpoint by activating Chk1 and/or Chk2[37,38]. The connection between NEK1 and DDR became more convincing by the findings that NEK1 kinase activity is required for ATR-ATRIP stability and ATR activation, which, in turn, activates Chk1[39]. Although NEK1 activity increases upon DNA damages, the upstream regulator of NEK1 was not reported before Singh *et al.*[27] published work. Singh *et al.*[27] reported that TLK1/1B phosphorylates NEK1 at a novel T141 residue that is adjacent to the activation loop and converts it to a hyperactive form. Since both TLK1/1B and NEK1 catalytic functions peak during the same phases of the cell cycle (S and G_2/M) and by DNA damage, it is likely that their activities are linked, i.e., TLK1/1B regulates the kinase activity of NEK1. Indeed, genetic depletion of NEK1 or overexpression of a NEK1 T141A variant or pharmacologic inhibition of TLK1/1B, all resulted in reduced activation of ATR and Chk1 and increased S phase duration, but decreased G_2/M phase cells suggesting the elimination of the cell cycle checkpoint after H_2O_2 treatment[27].

TLK1B MEDIATED COMPLEMENTATION OF THE AR/DDR ACTIVATION LOOP

Inhibition of AR signaling by ADT leads to the downregulation of multiple key DNA repair enzymes involved in homologous recombination repair (HRR), non-homologous end joining (NHEJ), and mismatch repair (MMR) pathways, resulting in inefficient DNA repair, which leads to genomic instability[9]. Several independent studies identified DNA-PKc, Ku70 (key players of NHEJ), BRCA1, RAD54L, RMI2 (associated with HRR), MSH2, MSH6 (key players of MMR) as direct targets of AR[5,7-9,40]. When cells try to undergo mitotic division with excessively damaged DNA, they become vulnerable to apoptotic death. These liabilities may render prostate cancer cells particularly sensitive to inhibition of specific DDR pathways, such as PARP in homologous recombination DNA repair[41] and Chk1 in cell cycle checkpoint and DNA repair[42], creating opportunities for synthetic lethality[43]. However, PCa cells may develop a compensatory mechanism by activating mTOR and translational upregulation of TLK1B to promote castration-resistant growth. Regulation via the translational increase in TLK1B isoform abrogates the need for its *de novo* gene expression, which is a faster and more energy-efficient mechanism of genome protection by activating TLK1>NEK1>ATR>Chk1 axis[30]. Singh *et al.*[30,31]. demonstrated that combination treatment of anti-androgen (bicalutamide) and TLK1 inhibitor [Thioridazine (THD)] synergistically decreases the formation of androgen-independent (AI) colonies of LNCaP cells and LNCaP xenograft tumors and increase apoptotic rate than bicalutamide treatment alone. We have yet to test in xenografts whether combination with second-generation anti-androgens, like enzalutamide, will produce the same effects, although, from the theoretical standpoint, there is no reason to suspect it would be different. Biochemically, ADT+THD treatment reduces pNEK1 T141, pATR, and pChk1 level, which suggest the possible involvement of TLK1>NEK1>ATR>Chk1 mediated DDR pathway in the development of CRPC. Similarly, NEK1 T141A overexpressing LNCaP and xenograft tumor remain sensitive to ADT and do not transition to AI growth, which confirms the persistence of TLK1>NEK1 signaling in PCa drug resistance to ADT[30]. Correlated increase of TLK1B and pNEK1 T141 with PCa progression was evident from a human PCa-TMA, TRAMP (a PCa mice model) and PDX tumor tissues; all suggested the universality of TLK1>NEK1 signaling in PCa models[31]. In fact, targeting TLK1 could potentiate the activity of other DNA damaging agents as evident from our group and others' works in different cancers. For instance, TLK1 inhibition chemosensitizes PCa (PC3) and breast cancer (MDA-MB-231 and 4T1) cells to doxorubicin (Ronald *et al.*[44]; Jin *et al.*[45]), glioblastoma cells (U87MG) to temozolomide (Ibrahim *et al.*[46]), cholangiocarcinoma (SSP25 and HuCCT1) and ovarian cancer cells (SKOV-3) to cisplatin (Takayama *et al.*[47]; Rho *et al.*[48]). Since resistance to cisplatin, doxorubicin, and radiotherapies are common in CRPC, TLK1 inhibition may reverse the resistance of CRPC cells to these agents[49,50]. Our group was first to identify a number of compounds that inhibit TLK1 and TLK2 in the class of phenothiazines antipsychotics, suggesting their repurposing as a clinical option[44], and a newer derivative (J54) lacking appreciable anti-dopaminergic activity[51].

ROLE OF TLK1 IN THE HIPPO/YAP PATHWAY

TLK1-NEK1 signaling has pleotropic effects on several oncogenic processes regulating PCa progression and drug resistance. A key finding from our lab is that TLK1 dependent hyperactive NEK1 can phosphorylate YAP and promote its stability[52]. The observation that NEK1 can interact and phosphorylate TAZ prompted us to investigate whether NEK1 can also phosphorylate YAP, a key effector of hippo pathway highly homologous to TAZ[53]. NEK1 genetic depletion or NEK1 T141A variant overexpression or TLK1 inhibition all resulted in YAP degradation and downregulation of YAP target genes in PCa cells. In addition, we demonstrated that NEK1 can phosphorylate YAP1 in six unique residues *in vitro*, of which pYAP1 Y407 seems most interesting[52]. YAP1 Y407 phosphorylation by NEK1 may increase the binding affinity of YAP to its transcriptional activators resulting in YAP nuclear translocation away from cytoplasmic degradation. Determining the functional relevance of these phosphorylation sites of YAP in CRPC progression and drug resistance development is currently an active pursuit of our lab. TLK1>NEK1>YAP axis may also converge with the reciprocal feedback loop of AR and DDR (AR signaling induces DNA repair genes expression and DNA damage increases AR activity), as YAP regulates AR target genes expression[7,54,55].

ROLE OF TLK1 IN OTHER PATHWAYS

TLK1-NEK1 axis regulates other survival pathways independent of DDR and hippo/YAP regulation. We demonstrated that TLK1>NEK1 axis can promote survival of PCa cells by maintaining mitochondrial membrane integrity by phosphorylating VDAC1 leading to apoptotic suppression[56]. VDAC1 is a pore-forming, outer mitochondrial membrane protein that regulates metabolic and ionic exchanges between the mitochondria and cell and is often overexpressed in high grade PCa (reviewed in Ref.[57]). NEK1 can phosphorylate VDAC1 on S193 residue controlling its gatekeeping activity[58]. We demonstrated that disruption of TLK1>NEK1 axis sensitizes the PCa cells to low doses of doxorubicin treatment by reducing VDAC1 S193 phosphorylation and its stability. Doxorubicin treatment of NEK1 T141A mutant variant overexpressing cells displayed enhanced population of sub G1 cells, reduced oxygen consumption, and increased cytochrome C (Cyt-C) release in cytoplasm resulting in the activation of intrinsic apoptotic pathway compared to the wild type NEK1 overexpressing cells[56].

TLK1 also directly facilitates other pro-survival pathways through some substrates independent of NEK1. TLK1 mediates anti-apoptotic responses through AKT activation by interacting and phosphorylating AKT interacting protein (AKTIP). TLK1 phosphorylation of AKTIP on T22 and S237 residues increases its scaffolding activity to anchor both AKT and PDK1 to the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) regions of the plasma membrane where PDK1 can phosphorylate AKT on T308 residue followed by S473 phosphorylation by mTORC2 complex for full activation of AKT[59]. Fully activated AKT may relay pro-survival signals by inhibiting BAD, caspase 9, and FoxO3 pro-apoptotic factors through phosphorylation facilitating CRPC progression. However, the role of AKTIP phosphorylation by TLK1 in CRPC drug resistance needs to be further assessed. In a recent endeavor, we uncovered the novel interaction between TLK1 and MK5 that promotes PCa cell motility and invasion. MK5 is a known promotility kinase that can be phosphorylated by TLK1 in three unique residues that increases MK5's catalytic function. We demonstrated that one phospho-acceptor site of MK5 (S354) by TLK1 is present in all major PCa cell lines of both androgen-dependent and independent categories. Furthermore, ADT increases pMK5 S354 level in LNCaP cells, and this pMK5 protein is progressively increased in tumors of higher grades and nodal metastatic scores analyzed from TRAMP mice prostate tissue and human PCa TMA, suggesting that the TLK1>MK5 signaling may be associated with increased aggressiveness of PCa. Finally, disruption of TLK1>MK5 axis by TLK1 or MK5 inhibition drastically reduces PCa cells motility analyzed in a panel of PCa cell lines[29]. Figure 1 graphically depicts the currently known multifaceted roles of TLK1 in mCRPC progression and acquisition of drug resistance.

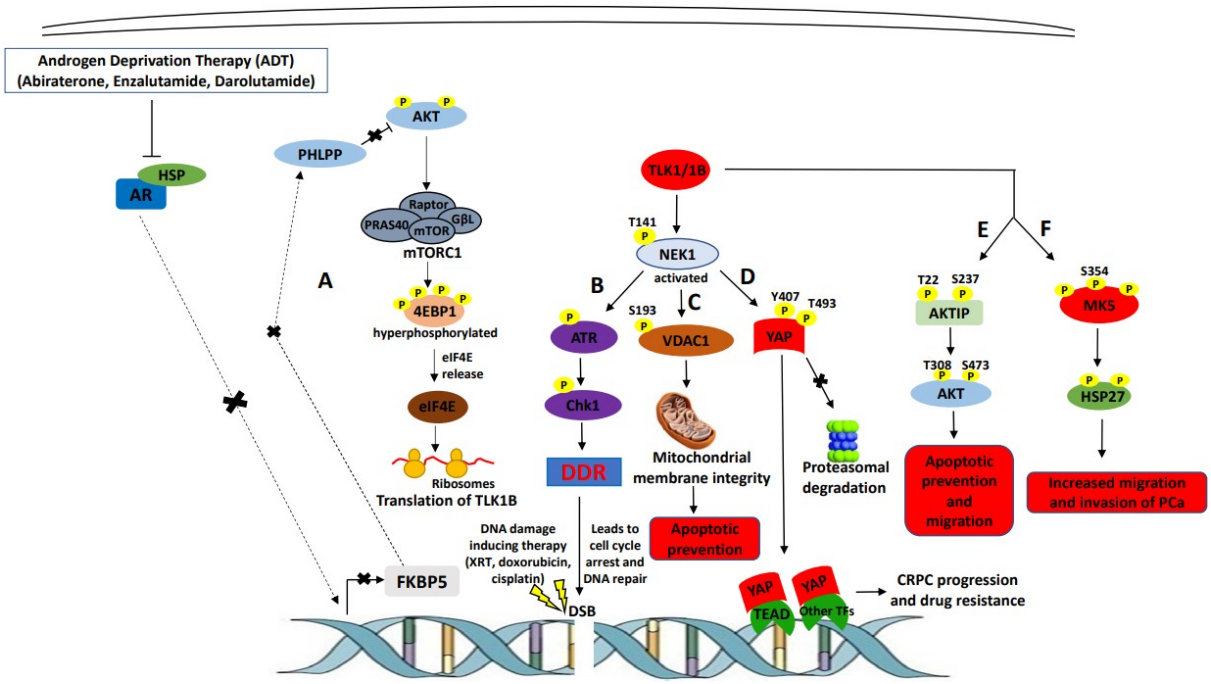

Figure 1. TLK1's role in mediating mCRPC progression and therapy resistance. (A) androgen deprivation therapy (ADT) blocks androgen receptor (AR) nuclear localization resulting in FKBP5 downregulation, which in turn, leads to AKT activation due to reduced dephosphorylation activity of PHLPP. AKT activation leads to mTORC1 activation, which activates 4EBP1 and releases eIF4E. Excess eIF4E initiates the translation of TLK1B. (B) TLK1/1B activates NEK1 by T141 phosphorylation, which in turn, activates ATR>Chk1>DNA damage response (DDR) signaling cascade. Activation of DDR promotes DNA repair inflicted by DNA damaging therapeutic agents. (C) TLK1>NEK1 axis is involved in VDAC1 S193 phosphorylation which maintains mitochondrial membrane integrity and suppresses intrinsic apoptotic signaling. (D) TLK1>NEK1 signaling phosphorylates YAP on Y407 and T493 residues and promotes the stabilization of YAP by binding to TEAD or other transcription factors (TF), leading to nuclear relocalization escaping proteasomal degradation. YAP stabilization and accumulation lead to CRPC progression and drug resistance. (E) TLK1 directly phosphorylates AKTIP on T22 and S237 residues, which stimulates AKT phosphorylation and activation, resulting in pro-survival and pro-migratory signaling. (F) TLK1 directly interacts and phosphorylates MK5, which increases its catalytic activity towards HSP27 (an MK5 substrate) and leads to increased migration and invasion, hence, metastasis of PCa cells.

TARGETING TLK1 AND THE DDR TO REDUCE DRUG RESISTANCE

DNA damage response (DDR) is a vital mechanism for PCa cells' survival and propagation. In addition, intact DDR activity may give hormone-refractory PCa cells an undue advantage against DNA damage-inducing therapies by keeping their genomic instability to sub-toxic level, thus, promoting drug resistance. Targeting TLK1 might be an excellent approach to impair DDR along with other oncogenic survival pathways as mentioned earlier. TLK1 is a better druggable target than other kinases of the DDR pathway, such as ATM or Chk1, to reduce general toxicity as ADT selectively increases TLK1B level in PCa cells, which can be easily manipulated using TLK1 specific inhibitors sparing the non-cancerous cells. Further efforts need to be given to design and generate better TLK1 inhibitors with improved pharmacokinetics, safety, and tolerability for clinical application. In fact, in recent years, our lab, in collaboration with others, has designed and synthesized a phenothiazine analog named J54 with higher efficacy in TLK1 inhibition and lower non-target effects unlike THD[51,52]. Personalized application of TLK1 inhibition as an adjuvant and neoadjuvant therapy in combination with other PCa directed treatments may overcome the drug-resistant phenotype of CRPC and bring therapeutic benefit.

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Authors' contributions

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Availability of data and materials

Description of data and materials can be found in the referenced article. No new data are included in this review.

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Cancer Drug Resistance

Review

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Tumor-derived exosomes: immune properties and clinical application in lung cancer

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Abstract

Lung cancer is the leading cause of cancer-related death worldwide. Despite advances in diagnosis and treatment of lung cancer, the overall survival remains poor. Evidence indicates that lung cancer development is a complex and dynamic process that involves interactions between tumor cells and their microenvironments, including immune cells. Exosomes are small extracellular vesicles secreted by most cell types; they contain functional molecules that allow intercellular communication. Tumor-derived exosomes (TEXs) carry both immunosuppressive and immunostimulatory mediators and may be involved in various immunomodulatory effects. TEXs, which partially mimic profiles of the parent cells, are a potential source of cancer biomarkers for prognosis, diagnosis, and prediction of response to therapy. In addition, TEXs may interfere with immunotherapies, but they also could be used as adjuvants and antigenic components in vaccines against lung cancer. In the context of lung cancer, identifying TEXs and understanding their contribution to tumorigenesis and the response to immunotherapies represents a challenging research area.

Keywords: Lung cancer, tumor-derived exosomes, cancer biomarkers, immunotherapies

INTRODUCTION

Lung cancer is one of the most common malignant tumors with the highest morbidity and mortality worldwide[1]. Recently, immunotherapies have shown more effectiveness than traditional chemotherapy,

and they have dramatically changed the treatment paradigm for lung cancer[2-4]. However, only a small proportion of patients can benefit from the immunotherapies, with primary and secondary resistance complicating treatment. One potential explanation for this phenomenon is the complexity and diversity of the tumor microenvironment (TME). Interactions of lung cancer cells with the surrounding TME are critical to cancer progression and response to immunotherapy. In recent years, the communication mediated by exosomes has extensively gained attention.

Exosomes are small bilayer membrane vesicles with a size of 30-100 nm in diameter that are secreted by various cell types such as tumor cells, immune cells, and fibroblasts[5,6]. Exosomes derived from tumor cells are referred to as tumor-derived exosomes (TEXs)[7]. TEXs have been shown to contain a variety of biomolecules including nucleic acids, proteins, enzymes, and lipids, which are involved in cancer progression, intercellular communication, and immunological function[8]. Studies have increasingly indicated that the number and composition of TEXs can in part reflect their cells of origin and biological state, which may serve as potential biomarkers in diagnosis and prognosis of cancer[9-11]. TEXs may affect cancer immunotherapy either by sequestration of therapeutic antibodies or supplying self-antigen carriers to improve cancer vaccine efficacy. Their biological roles in cancer progression as well as cancer immunotherapy and biomarkers have indicated that TEXs are critical components of the TME.

In this review, we first describe how TEXs are formed and released to the extracellular matrix, and discuss the composition of TEXs. Then, we outline the immunomodulatory function of TEXs in the lung cancer microenvironment. Moreover, we focus on the utility of TEXs as diagnostic and prognostic biomarkers in lung cancer. Finally, the recent findings on TEXs in immunological changes during immunotherapy are discussed.

THE FORMATION, RELEASE, AND COMPOSITION OF TEXS

TEXs, released by tumor cells, are present ubiquitously in tumor tissues and body fluids[12]. Exosome biogenesis initiates from the production of early endosomes via the internalization of membrane microdomains. The limiting membrane of early endosomes bud inwardly to form intraluminal vesicles, then becoming the multivesicular bodies. Finally, exosomes are released when multivesicular bodies fuse with the plasma membrane[13]. This formation of exosomes is a tightly regulated process; it involves two pathways through an Endosomal Sorting Complex Required for Transport (ESCRT)-dependent machinery or an ESCRT-independent machinery[14]. Once exosomes released, they are able to transfer information to their recipient cells through three main ways: endocytosis/phagocytosis, direct fusion with cellular membrane, and receptor-ligand interactions[13] [Figure 1A].

TEXs consist of a lipid-protein bilayer membrane, including membrane transport and fusion proteins (e.g., annexins, Rab proteins, and flotillin), MHC (class I and II molecules), adhesion molecules (e.g., ICAM, EPCAM, CD44, and integrins), inhibitory ligands [e.g., FasL, TRAIL, PD-L1, and transforming growth factor (TGF)-β/LAP], tetraspanins (e.g., CD9, CD63, CD81, and CD82), tumor associated antigens, chaperones [e.g., heat-shock protein (HSP) 70 and HSP90], lipids, and glycolipids[12,13,15-17]. In their lumen, TEXs carry a variety of multivesicular bodies biogenesis proteins [e.g., Alix and tumor susceptibility gene 101 (TSG101)], cytoskeletal proteins (e.g., actin, tubulin, and vimentin), histones, oncoproteins, soluble factors, enzymes, cytokines, signaling molecules, and nucleic acids (including DNA, mRNA, and miRNA)[13,15] [Figure 1B]. The TEXs molecular and genetic content mimics that of parent cells, and is in part considered as surrogates of the parent tumor cells[18]. Moreover, TEXs can transfer messages from the parent tumor to recipient cells, including immune cells, within the TME[19].

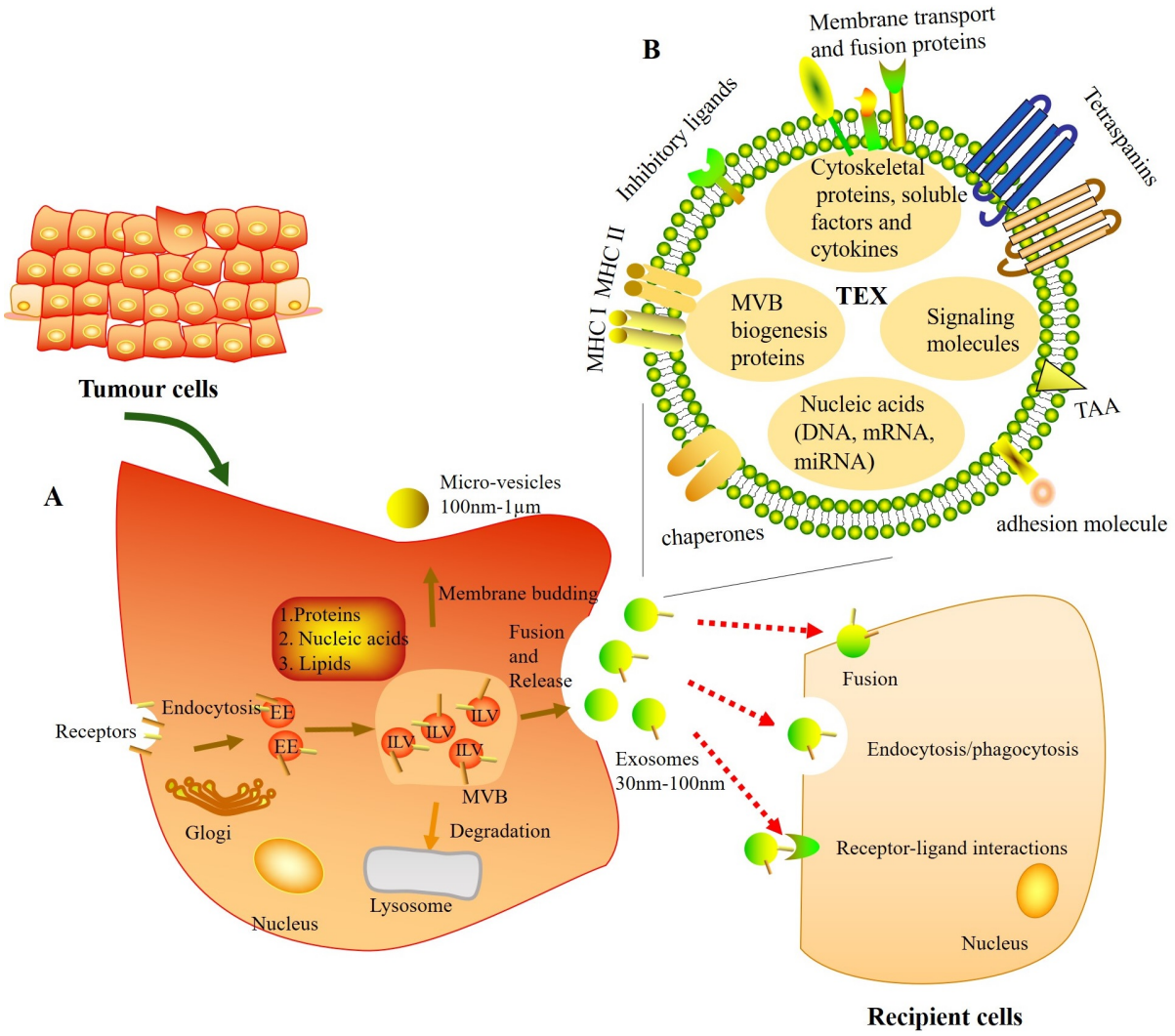

Figure 1. Molecular composition, biogenesis, release, and uptake of tumor-derived exosomes (TEXs). (A) TEXs originate from intraluminal vesicles (ILVs) in the multivesicular bodies (MVBs) (also known as late endosomes). Firstly, early endosomes (EEs) are formed when the membrane microdomains are endocytosed via inward budding of the plasma membrane. Then EEs mature into MVBs, which follow either fusion with the plasma membrane to form exosomes or degradation by lysosome. During this process, the proteins, nucleic acids, and lipids are packed into exosomes. Finally, exosomes can interact with recipient cells through three main ways: endocytosis/phagocytosis, direct fusion with cellular membrane, and receptor-ligand interactions. (B) Schematic diagram of components of TEXs.

EFFECT OF TEXS FOR IMMUNE REGULATION IN LUNG CANCER

Recently, TEXs have been proposed to act as crucial mediators between cellular communication by transferring both immunosuppressive and immunostimulatory signals to immune cells in a lung cancer microenvironment[20,21] [Table 1, Figure 2].

Immunosuppressive effect of TEXs

TEXs modulate the activity of T cells by promoting apoptosis and inhibiting proliferation of $CD8^+$ T cells[22]. Recent studies have indicated that TEXs contain PD-L1, which inhibits T cells activity and promotes tumor progression. Exosomes from lung cancer, melanoma, and breast cancer carry PD-L1 on their surface, which interacts with PD-1 via the extracellular domain, and thereby inactivate T cells[23]. Poggio *et al.*[24] discovered that the majority of PD-L1 could be presented on the surface of TEXs, and genetic blockade of exosomal

Table 1. Overview of exosomal cargo, source of exosomes, and their biological effects

Exosomal cargo	Donor	Biological effect	Ref.
PD-L1	H1299, H358, and H1264	Inactivate T cells	[23]
EGFR	NSCLC biopsies	Induce tolerogenic DCs	[28]
miR-214	Lewis lung carcinoma cells	Downregulate the PTEN-mediated signaling	[29]
miR-433	Plasma of NSCLC patients	Inactivate the WNT/β catenin signaling	[31]
miR-21/29a	A-549 and SK-MES	Activate TLR7 and TLR8	[32]
miR-21a	Lewis lung carcinoma cells	Promote MDSCs expansion	[51]
miR-103a	CL1-5 lung cancer cells	Activate of PI3K/Akt and STAT3 signaling pathways	[52]
circFARSA	A549 and PC9 cells	Polarize macrophages to the M2 phenotype	[54]
miR-770	A549 cells	Suppress M2 macrophage polarization	[62]

NSCLC: Non small cell lung cancer; DCs: dendritic cells; MDSCs: myeloid-derived suppressor cells.

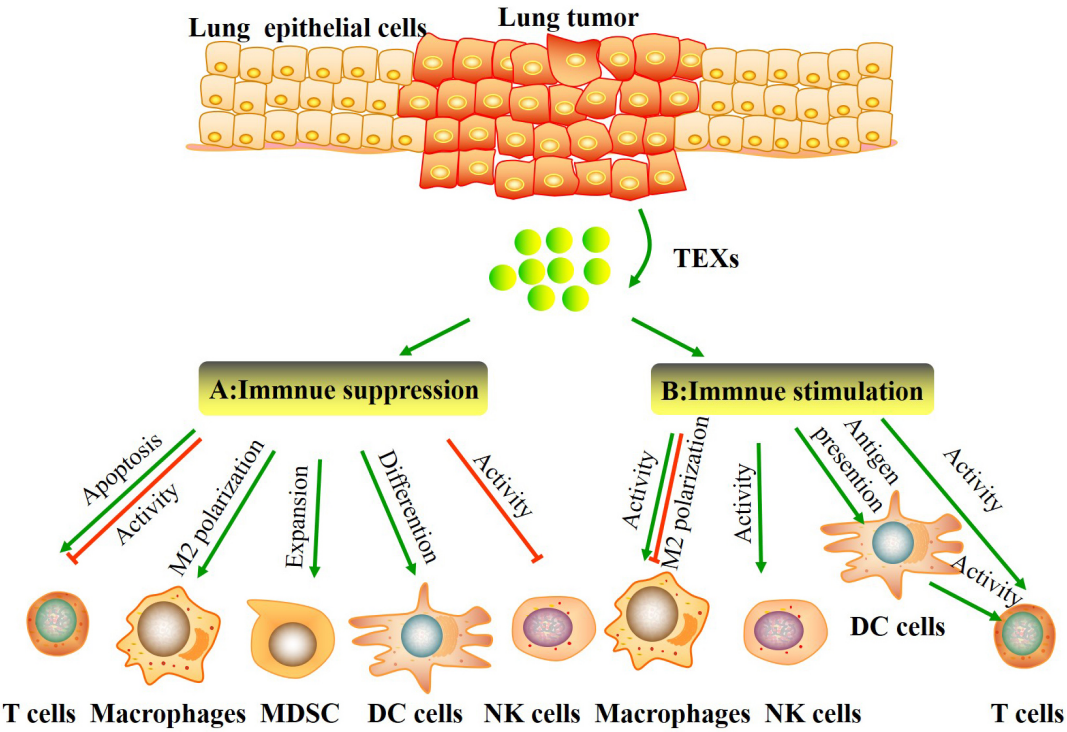

Figure 2. Tumor-derived exosomes (TEXs) carry and deliver both immunosuppressive and immunostimulatory signals to immune cells in the lung tumor microenvironment. (A) Immune suppression. TEXs contribute to establish an immunosuppressive TME by inducing apoptosis and inhibiting the activity of effector T cells, skewing M2 polarization of macrophages, expanding myeloid-derived suppressor cells (MDSCs), suppressing DCs differentiation, and impairing the function of NK cells. (B) Immune stimulation. TEXs can also stimulate immune cells to support antitumor activities, including enhancing the activity of macrophages and NK cells, suppressing M2 macrophage polarization, and increase T cells activity directly or indirectly.

PD-L1 could activate an anti-tumor immune response leading to extend survival in a subset of cancer patients. Moreover, it is suggested that TEXs with FasL expression could induce $CD8^+$ T cell apoptosis[25,26]. Czystowska *et al.*[27] uncovered the mechanism that the PI3K/Akt pathway was a central target for TEXs in regulating $CD8^+$ T cell apoptosis. Huang *et al.*[28] indicated that about 80% of exosomes isolated from non-small cell lung cancer (NSCLC) biopsies contained EGFR. These exosomes can be captured by dendritic cells (DCs). Then tolerogenic DCs were generated and induced tumor antigen specific regulatory T cells (Tregs), which could inhibit the function of tumor specific $CD8^+$ T cells. Yin *et al.*[29] found that miR-214

was delivered into recipient $CD4^+$ T cells via TEXs, so as to downregulate the PTEN-mediated signaling, thereby promoting Treg expansion and tumor growth. Interestingly, co-incubation of Treg with TEXs may enhance Treg number as well as its suppressive function with the increased production of inhibitory cytokines, TGF-β1 and interleukin (IL)-10[30]. Additionally, Liu *et al.*[31] showed that exosome-derived miR-433 inactivated the WNT/β-catenin signaling pathway via targeting transmembrane p24 trafficking protein 5, thus increasing infiltration of $CD4^+$ and $CD8^+$ cells in NSCLC.

Exosome-derived miR-21/29a derived from A-549 and SK-MES cells promoted lung cancer growth and metastasis through activating Toll like receptors TLR7 and TLR8 on immune cells including NK cells[32]. NK cells express a variety of receptors that are either stimulatory or inhibitory[33]. The downregulation of those stimulatory receptors, particularly NKG2D, may play an important role in decreasing activity of NK cells in lung cancer patients[34]. TEXs originating from hypoxic tumor cells deliver TGF-β1 to NK cells, and thereby reduce NKG2D expression resulting in lower activity of NK cells[35]. TEXs can also attenuate NK cell activity via multiple mechanisms including shedding the NKG2D ligand on tumor cells, suppressing Janus kinase (Jak) 3 activation, inhibiting perforin or cyclin D3 production and down-regulation of IL-2-mediated pathways[36-41]. Moreover, TEX-carried MICA and MICB ligands can downregulate the stimulatory receptors, especially NKG2D on NK cells[42].

TEXs suppressed the functioning of the immune system by affecting the monocyte differentiation and maturation[43,44]. TEXs were capable of blocking DCs migration to lymph nodes through inhibiting most C-C/C-X-C chemokine receptor expression[45]. Co-incubation of DCs with TEXs leaded to the down regulation of CD80 and CD86, and subsequently inhibited DCs maturation[46]. In addition, TEXs have been shown to induce the differentiation of myeloid precursor cells into highly suppressive myeloid-derived suppressor cells (MDSCs)[39,47]. Heat-shock protein 72 (Hsp72) on the TEXs surface could trigger the STAT3 activation and autocrine IL-6 production in MDSCs via a TLR2/MyD88-dependent manner, leading to the increment of immunosuppressive activity of MDSCs[48-50]. Zhang *et al.*[51] indicated that miR-21a enriched in lung carcinoma cell-derived exosomes could promote MDSCs expansion via targeting PDCD4, thus enhancing tumor growth. Hsu *et al.*[52] reported that miR-103a was upregulated in lung cancer derived exosomes under hypoxic conditions. Exosomal miR-103a could cause the activation of PI3K/Akt and STAT3 signaling pathways by directly targeting PTEN, thereby resulting in the enhancement of M2 polarization[52]. Additionally, the study with A549 (wild-type p53 allele) and H358 (p53 null allele) exosomes suggested that lung cancer cell-derived exosomes mediated M2 polarization may be p53 independent[53]. Another study showed that exosomal circFARSA was significantly upregulated in NSCLC tissues and stimulated NSCLC cell metastasis by polarizing macrophages to the M2 phenotype[54].

Taken together, these data suggested that TEXs can modulate the immune response by transferring immunosuppressive signals to immune cells, which in turn contribute to tumor progression[7,34,55,56].

Immunostimulatory effect of TEXs

TEXs have been reported to be involved in the suppression of the immune system in previous studies. However, since TEXs also carry stimulatory molecules that contribute to activating immune responses, recent studies also focused on anti-tumor immunity of exosomes.

TEXs can act as presenters participating in direct and indirect antigen presentation[57]. As direct presenters, TEXs present antigen to T cells via an MHC-peptide complex on their surface. On the other hand, TEXs can also indirectly transfer tumor antigen to antigen presenting cells, like DCs, and then activate the cytotoxic activity of $CD8^+$ T cells and $CD4^+$ T helper cells, so as to inhibit tumor growth[58]. Additionally, the

enrichment of HSPs on TEXs such as Hsp70, can stimulate the activity of NK cells[59] and macrophages[60], and induce MHC class I-restricted cytotoxic T cells activation[61]. Tumor cell-derived exosomal miR-770 could suppress M2 macrophage polarization via targeting MAP3K1, which in turn decreased NSCLC tumor growth[62]. Tetraspanins on the exosome surface mainly mediate cell adhesion and participate in maintaining the optimal conformation of immune proteins like MHC class II, thus playing an important role in antitumor immunity through exosomal targeting to DCs[61,63-65].

Therefore, the effect of exosomes' immune stimulation depends mainly on their antigen presentation, while the effect of inhibiting immunity mainly depends on exosome-carried biological content consisting of ligands, miRNAs, and proteins, which may inhibit the cytotoxic activity of the NK and $CD8^+$ T cells or increase suppressive immune cells such as MDSCs, Treg cells, and M2 macrophages. Understanding the effect of TEXs in immune regulation will allow for better understanding of the clinical application of TEXs in cancer diagnosis and treatment.

TEXS SERVE AS DIAGNOSTIC AND PROGNOSTIC BIOMARKERS IN LUNG CANCER

TEXs and their content in biofluids, which represent the content of parent cells, may serve as newly developed non-invasive biomarkers for diagnosis, prognosis, and monitoring the efficacy of treatment in lung cancer[9-11].

Jakobsen *et al.*[66] examined the potential of exosomal proteins as diagnostic markers in advanced NSCLC. The EV (extracellular vesicle) array showed that the expression levels of CD9, CD63, and CD81 were significantly high in cancerous patients. Likewise, according to the EV array, NYESO-1, EGFR, and PLAP showed a strong correlation with a poor survival in NSCLC[11]. In addition, SRGN, TPM3, THBS1, and HUWE1 may serve as biomarkers to distinguish lung adenocarcinoma subjects from controls[67]. Combination of carcinoembryonic antigen, exosomal alpha-2-HS-glycoprotein and extracellular matrix protein 1 (ECM1) could improve the diagnostic accuracy of NSCLC[68]. Gao *et al.*[69] indicated that plasma exosomal total protein, Tim-3 and Galectin-9 were significantly increased in NSCLC, and were positively associated with larger tumor size, advanced TNM stage, and distant metastases. The higher level of leucinerich a-2-glycoprotein (LRG1) was detected in urinary exosomes and may be a non-invasive diagnostic biomarker of NSCLC in urine[70].

Several studies have shown that exosomal miRNAs may serve as potential biomarkers for the early diagnosis of lung cancer. Rabinowits *et al.*[71] suggested that exosomal miRNAs in NSCLC patients very closely resemble those in NSCLC tissue, indicating that such a liquid biopsy may obviate the need to obtain tumor tissues. Tumor-derived exosomal miRNAs, adenocarcinoma-specific miR-181-5p, miR-361-5p, miR-30a-3p, and miR-30e-3p, and squamous cell carcinoma-specific miR-10b-5p, miR-320b, and miR-15b-5p, were isolated from the plasma of early-stage NSCLC patients, which are able to discriminate between adenocarcinoma and squamous cell carcinoma, thereby serving as noninvasive biomarkers for early diagnosis of NSCLC[72]. Exosomal miR-1169 and miR-260 have also been identified as potential biomarkers that can distinguish between early-stage wild-type EGFR and mutant EGFR NSCLC[73]. Exosomal miR-20b-5p and miR-3187-5p were drastically reduced in early-stage NSCLC patients than those in healthy controls, showing both exosomal miRNAs were efficient diagnostic biomarkers for early-stage NSCLC[74]. Exosomal miR-126 has also been identified as a possible diagnostic biomarker for NSCLC progression[75]. In fact, exosomes exist extensively in body fluids other than blood, and increasing attention has been focused on diagnostic assays in pleural fluid. Tamiya *et al.*[76] revealed that exosomal miR-182 and miR-210 were demonstrated to have a diagnostic potential for differentiating lung adenocarcinoma pleural effusion from benign pleural effusion. Additionally, exosomal miR-200 and mRNA transcript encoding lipocalin-2 from

pleural effusions may be considered as diagnostic markers to discriminate lung adenocarcinoma patients from patients with benign inflammatory processes[77].

Recently, exosomal RNAs have been reported to predict the prognosis of a variety of cancers including lung cancer. Dejima *et al.*[78] showed that exosomal miR-21 and miR-4257 levels of the NSCLC patients were significantly upregulated during recurrence and can be used as recurrence-specific biomarkers. Additionally, another study reported that low exosomal let-7a-5p levels were significantly associated with a worse cancer-related survival rate in lung adenocarcinoma patients[79]. Luo *et al.*[80] also demonstrated that serum exosomal miR-382 was considered as an independent prognostic biomarker for NSCLC. Zhang *et al.*[81] suggested that exosomal lncRNA MALAT-1 was highly expressed in NSCLC patients and was positively associated with lymphatic metastasis and TNM stage, thereby indicating that exosomal MALAT-1 may be a non-invasive biomarker for diagnosis and prognosis of NSCLC. Moreover, tumor-derived exosomal eIF4E has the potential for use as a biomarker for survival prediction in NSCLC[82].

Acquired resistance to general therapies, including chemotherapy, radiotherapy, immunotherapy, and targeted therapy, is a major challenge in the treatment of lung cancer. Nowadays, the use of exosomes as biomarkers for predicting therapeutic responses has gathered much attention. Exosomal hsa_circ_0014235 isolated from plasma promoted cisplatin chemoresistance and may serve as a promising biomarker for NSCLC treatment[83]. Exosomal miR-4443 might also promote cisplatin resistance of NSCLC by regulating FSP1-mediated ferroptosis[84]. In addition, Li *et al.*[85] showed that plasma exosomal miR-92b-3p was significantly increased in chemoresistant small cell lung cancer patients and might serve as a potential dynamic biomarker for monitoring the drug resistance. Exosomal miR-29a-3p and miR-150-5p were identified as circulating biomarkers during thoracic radiation therapy for NSCLC and were correlated with delivered radiation therapy dose[86]. At present, precision medicine based on immunotherapy and targeted therapy has given new hope for lung cancer patients. The ensuing problems of drug resistance have gained much interest from the research community. For example, exosomal miR-323-3p, miR-1468-3p, miR-5189-5p, and miR-6513-5p were identified as potential biomarkers to discriminate between osimertinib-resistant and osimertinib-sensitive NSCLC patients[87], and exosomal miR-210-3p secreted by osimertinib-resistant HCC827 and PC-9 cells was able to promote epithelial-mesenchymal transition and drug resistance in osimertinib-sensitive, EGFR mutant NSCLC cells[88]. Moreover, exosomal circRNA_102481 enhanced EGFR-TKIs resistance through the microRNA-30a-5p/ROR1 axis in NSCLC[89]. Peng *et al.*[90] suggested that high exosomal miR320d, miR320c, and miR320b levels were corelated with poor response to anti-PD-1 treatment in patients with NSCLC, and exosomal miR-125b-5p was identified to be one potential target for anti-PD-1 treatment. In summary, exosomal RNAs in lung cancer could be used for monitoring therapy response/relapse to improve personalized therapy strategies.

THE ROLE OF TEXS IN IMMUNOTHERAPY OF LUNG CANCER

Effect of TEXs for resistance to immunotherapy

TEXs may suppress proliferation and differentiation of immune cells and have the ability to influence their biological function. TEXs carrying a variety of tumor associated antigens or immunoinhibitory mediators not only suppress antitumor functions of immune effector cells, but also appear to impede effective response to immunotherapy in cancer[8]. Tumor associated antigens on TEXs could efficiently bind antibodies produced against cancer cells and block the access of therapeutic antibodies to the cancer cells, leading to a decrease in effectiveness of cancer therapy[91]. Additionally, TEXs are able to inhibit antibody dependent cell-mediated cytotoxicity, which serves as a critical mechanism of therapeutic antitumor activity of anticancer antibodies[91]. During therapy, immune escape in NSCLC occurs through a multistep process that facilitates tumor growth and progression. Acquired tumor resistance to immunotherapy could be

directly reflected in the production of TEXs[92]. Most importantly, Kim *et al.*[93] showed that lung cancer cells increased their production of immunosuppressive exosomes during acquired resistance to anti-PDL1 immunotherapy.

TEXs-based cancer vaccine and immunotherapy

TEXs are nanoscale membrane-derived vesicles that are thought to be important mediators of intercellular communication. Moreover, TEXs with distinct characteristics such as stability, permeability, biocompatibility, low immunogenicity, and low toxicity can efficiently deliver tumor antigens to DCs, thus they can be used as self-antigen carriers to stimulate immune response[94-96]. Increasing evidences have demonstrated that the activation and maturation of DCs by TEXs could enhance anti-tumor effects and may be applied for lung cancer immunotherapy. For example, TEXs from *CD40L*-gene modified 3LL lung tumor cells have the potent ability to activate DCs, resulting in significantly increased tumor antigen-specific $CD4^+$ T cell proliferation and $CD8^+$ T cell responses, revealing a powerful antitumor effect[97]. In addition, the exosomes derived from Rab27-overexpressing NSCLC cells also stimulated the proliferation and maturation of DCs effectively, promoted $CD4^+$ T cell proliferation and elicited potent antitumor immune responses[98]. Multiple studies have already proved that TEXs which were used as tumor antigens source for DC vaccines, have greater efficacy and safety than conventional tumor cell lysates[99-103]. Wang *et al.*[104] indicated that TEXs were more potent than tumor cell lysates to trigger DC-mediated immune responses and decrease Tregs, contributing to improving vaccine-elicited immunotherapy for lung cancer. Thus, DCs loaded with TEXs may be promising therapeutics without severe side effects and treatment resistance in clinical application[105].

CONCLUSION

TEXs are important mediators of intercellular communication and have been proven to play a key role in the TME. The biogenesis and secretion of TEXs have been widely reported. They carry a variety of cargoes and are involved in both immunosuppressive and immunostimulatory signaling pathways by delivering molecular signals to immune cells. The small size of TEXs and their contents render them highly interesting for biomedical applications, such as biomarker molecules and anticancer vaccines. Based on the data herein, we suggest that TEXs could be manipulated to provide clinical benefits and improve the clinical management of lung cancer.

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Authors' contributions

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Study design: Zhang P
All authors read and approved the final manuscript.

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Cancer Drug Resistance

Opinion

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Immunotherapy resistance of lung cancer

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Abstract

In recent years, immunotherapy has made remarkable breakthroughs and brought long-term survival benefits to lung cancer patients. However, a high percentage of patients do not respond to immunotherapy or their responses are transient, indicating the existence of immune resistance. Current studies show that the interactions between cancer cells and immune system are continuous and dynamic. A range of cancer cell-autonomous characteristics, tumor microenvironment factors, and host-related influences account for heterogenous responses. Furthermore, with the identification of new targets of immunotherapy and the development of immune-based combinations, we propose the response strategies to overcome resistance.

Keywords: Immunotherapy, resistance mechanisms, response strategies

INTRODUCTION

According to the latest cancer report of China, the morbidity and mortality of lung cancer are still the highest among all types of malignancies. Immune checkpoint inhibitors (ICIs) have dramatically changed the treatment landscape for advanced non-small cell lung cancer (NSCLC). In neoadjuvant therapy, ICIs combined with chemotherapy have increased the chances of cure for patients with early-stage NSCLC. In maintenance treatment, durvalumab significantly prolonged the disease-free survival time of NSCLC after concurrent radiotherapy and chemotherapy. The five-year survival rate of patients with advanced NSCLC

who received ICI monotherapy increased from 5% to 15%. At the same time, ICIs combined with chemotherapy have also brought a giant breakthrough in the treatment of advanced small cell lung cancer (SCLC). However, the majority of patients treated with ICIs are either non-responders or eventually develop progressive disease[1,2]. Therefore, clarifying resistance mechanisms and proposing response strategies for immunotherapy are ongoing challenges that need to be coped with. The tumor is a heterogeneous and dynamic tissue, which continuously evolves in the attempt to overcome structural, metabolic, and immunologic hurdles. Thus, resistance mechanisms and pathways are intertwined. Generally, the resistance mechanism of immunotherapy can be classified into two categories: intrinsic mechanisms and extrinsic mechanisms. Intrinsic mechanisms refer to the genetic, transcriptional or functional profile of the tumor cells themselves, while the extrinsic mechanisms refer to the tumor microenvironment and factors other than the tumor itself. The changes of immunosuppressive cells, immunosuppressive cytokines, coinhibitory receptors, and costimulatory receptors in the tumor microenvironment can destroy the antitumor immune response, mediating resistance to immunotherapy. Host-related factors including physical status, previous comorbidities, distribution of intestinal flora, and use of antibiotics could also affect the efficacy of immunotherapy. This opinion article intends to discuss the currently known mechanisms of immune resistance and potential response strategies in lung cancer. The mechanisms and factors of immune resistance discussed in this opinion are summarized in Figure 1.

RESISTANCE MECHANISMS OF IMMUNOTHERAPY IN LUNG CANCER

Intrinsic mechanisms and factors of resistance

The intrinsic factors and mechanisms of resistance mainly refer to the expression or inhibition of specific genes and pathways of tumor cells, such as the activation of driver genes and the inactivation of suppressor genes, low tumor mutation burden (TMB), decreased tumor antigen presentation, and changes in the expression of programmed cell death ligand 1 (PD-L1) of tumor cells.

Activation of driver genes

Genomic or epigenetic alternations in tumor cells can mediate immune escape. Several driver genes have been identified in lung cancer, such as epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and Kirsten rat sarcoma virus oncogene homolog (KRAS). They have all been confirmed to be related to the process of immune escape.

EGFR mutation is the most common type of mutation of NSCLC in Asians, with an incidence of 40.3%-64.5%[3]. Clinical trials showed that these patients do not respond well to immune checkpoint monotherapy[4-6]. There are several hypotheses for the inferior response of immunotherapy in this group of people. First, the immune microenvironment of this population belongs to the immune desert or immune excluded type with poor outcomes of immunotherapy[7,8]. After the activation of EGFR pathway, the ligand amphiregulin (AREG) could promote the production of regulatory T (Treg) cells to enhance the immunosuppressive effect through EGFR-glycogen synthase kinase-3β (GSK-3β)-forkhead box P3 (FOXP3) axis[9]. Besides, the EGFR signaling pathway can also produce inhibitory cytokines, induce myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) proliferation, and inhibit CD8+ T Cell response[10]. Second, the signal transducer and activator of the transcription 3 (STAT3) from the downstream of the signal pathway is upregulated, which in turn leads to the decreased expression of major histocompatibility complex I[11]. STAT3 also mediates the expression of vascular endothelial growth factor (VEGF), interleukin-6 (IL-6), and interleukin-10 (IL-10) and inhibits the differentiation and maturation of dendritic cells (DC). These changes will ultimately affect the presentation of antigens and the production of new antigens. Third, although most studies have demonstrated that EGFR will upregulate PD-L1, due to the decrease of new antigens and the increase of immunosuppressive cells, patients have not received durable

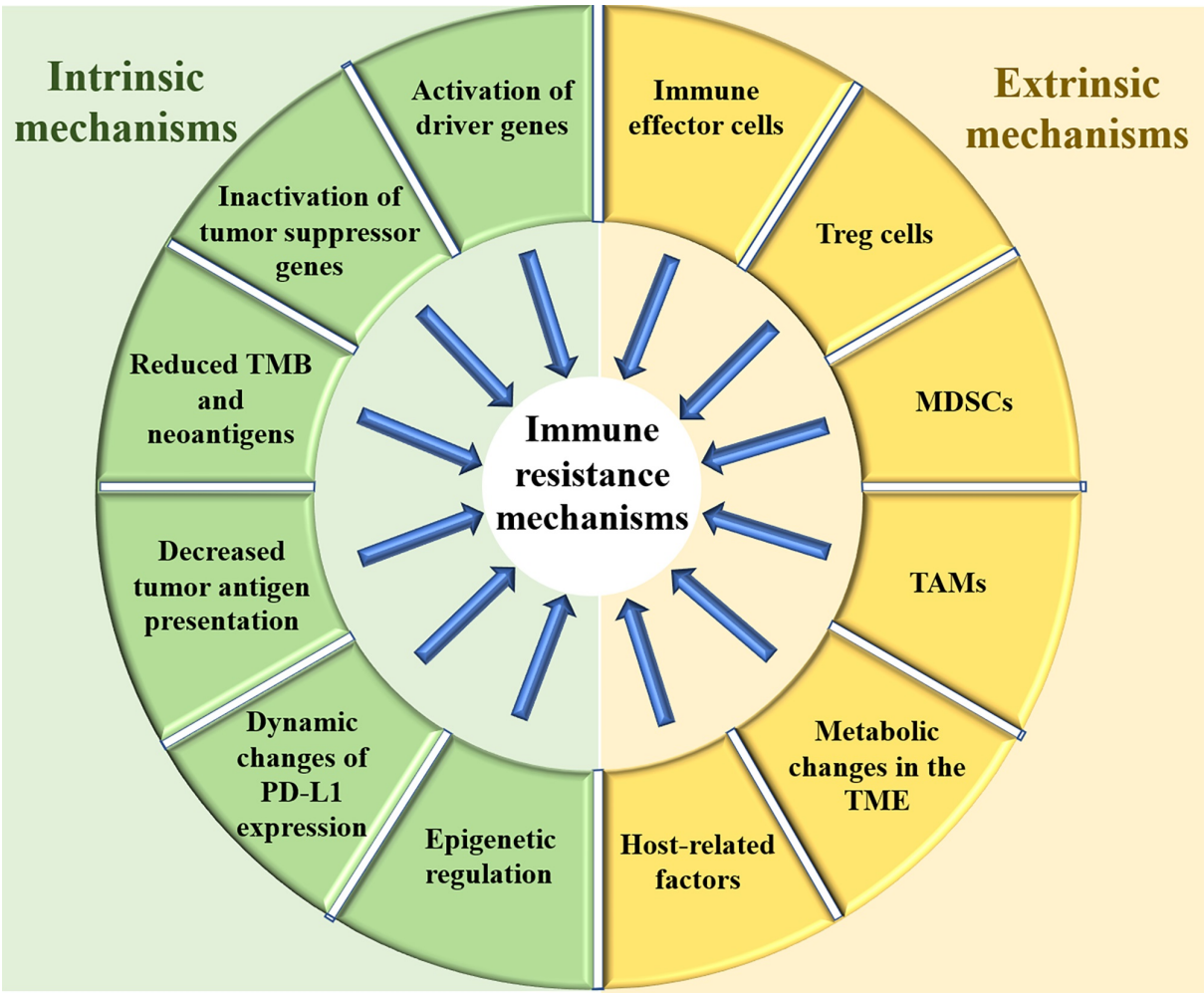

Figure 1. The intrinsic and extrinsic mechanisms and factors of immune resistance. TMB: Tumor mutation burden; PD-L1: programmed cell death ligand 1; MDSCs: myeloid-derived suppressor cells; TAMs: tumor-associated macrophages; TME: tumor microenvironment.

benefits from immune checkpoint monotherapy[10].

Another common gene mutation in lung cancer is ALK fusion. ALK fusion can reduce the production of new antigens and increase the number of immunosuppressive cells through the PI3K-AKT and MEK-ERK pathways, resulting in poor efficacy of immune checkpoint monotherapy[12,13]. *KRAS* is another common driver gene in lung cancer. Studies showed that KRAS mutation could increase the expression of PD-L1 to promote immune escape by regulating the stability of the 3'UTR region of PD-L1 mRNA[14]. However, KRAS mutations are correlated with an inflammatory tumor microenvironment and tumor immunogenicity, resulting in superior patient response to PD-1/PD-L1 inhibitors. In a single-center retrospective study which included 25 cases of KRAS mutations and 47 wild-type cases, patients were administered with nivolumab ± anti-CTLA-4 antibody. The median overall survival (OS) of the two groups was 18.1 and 8.1 months, respectively (HR = 0.48, P = 0.04)[15]. Mechanically, KRAS mutations induce the expression of neoantigens and change the expression a series of genes, such as cell cycle regulation, DNA replication, and DNA repair, but they have no obvious effect on activating immunosuppressive cells[16]. Several driver mutations of NSCLC and their chances to receive ICI therapy are summarized in Table 1.

Table 1. Common driver mutations of NSCLC and their chances to receive ICI therapy

EGFR mutation		ALK mutation		KRAS mutation			Other mutations
ICI monotherapy	ICI combination therapy	ICI monotherapy	ICI combination therapy	Single mutation	KP co-mutations	KL co-mutations	MET/RET/ROS1/BRAF/HER2
×	√	×	?	√	√	×	?
Not recommended	Consider using ICIs in some cases	Not recommended	Consider using ICIs in some cases	Probably benefit from ICIs	Probably benefit from ICIs	Not recommended	No large-scale trials and need further research

NSCLC: Non-small cell lung cancer; ICIs: immune checkpoint inhibitors; EGFR: epidermal growth factor receptor; ALK: anaplastic lymphoma kinase; KRAS: Kirsten ratsarcoma viral oncogene homolog; KP: KRAS/TP53; KL: KRAS/STK11; MET: mesenchymal to epithelial transition factor; RET: ret proto-oncogene; ROS1: c-ros oncogene 1; BRAF: v-raf murine sarcoma viral oncogene homolog B; HER2: human epidermal growth factor receptor-2.

Inactivation of tumor suppressor genes

The inactivation of tumor suppressor genes, such as *PTEN* and *STK11* genes, can also lead to poor outcomes of immunotherapy for lung cancer patients. In drug-resistant populations, the occurrence of the tumor suppressor PTEN mutations are significantly increased[17]. PTEN is a lipid phosphatase that inhibits the activity of the PI3K pathway. Current studies have shown that loss of PTEN expression is the most common way to activate the PI3K pathway in multiple cancers[18]. The PI3K pathway plays an indispensable role in tumor growth by regulating tumor proliferation and survival. Another study found a decreased expression of tumor-infiltrating lymphocytes (TILs) in patients with a high deletion rate of *PTEN* gene[19]. In addition, PTEN can upregulate the expression of VEGF and prevent tumor immune escape mediated by lymphocyte infiltration. *STK11/LKB1* is another tumor suppressor gene. About 8%-39% of patients with NSCLC have *STK11* gene mutation, which has the function of negatively regulating the rapamycin signal pathway. *STK11* gene mutations are often accompanied by a decreased infiltration of cytotoxic CD8+ T lymphocytes, leading to the "cold" immune microenvironment[20]. Co-mutations of KRAS and STK11 (KL) or TP53 (KP) could also affect the efficacy of ICI therapy. Skoulidis *et al.*[21] reported that patients with KRAS mutations and STK11/LKB1 alterations had reduced efficacy to immunotherapy in a cohort of 165 patients diagnosed with KRAS-mutated lung adenocarcinoma who had received PD-1/PD-L1 inhibitors treatment. In a mice model of KRAS mutation, the deletion of *STK11* gene promotes the resistance of PD-1 inhibitors monotherapy. Thus, KL tumors show resistance to PD-1 inhibitors, while KP tumors are more sensitive. Besides, the mutation rate of *SMARCA4* gene in NSCLC is about 10%, which is responsible for encoding the catalytic unit of the SWI/SNF chromatin remodeling complex. A study included 2690 NSCLC patients, of whom 211 patients carried SMARCA4 mutations[22]. Further analyses revealed that KRAS and SMARCA4 co-mutations are the most prevalent type. Among the NSCLC patients harboring KRAS mutations, patients with the co-occurrence of SMARCA4 mutation had a reduced objective response rate (ORR) (0.0% *vs.* 23.5%, $P = 0.02$), progression-free survival (PFS) (1.7 months *vs.* 4.1 months, $P < 0.001$), and median OS (4.0 months *vs.* 14.9 months, $P < 0.001$). In summary, mutations of driver genes and tumor suppressor genes can lead to changes in tumor neoantigens and can also alter the composition of the immune microenvironment, which is reflected in the heterogeneous efficacy of immunotherapy. Thus, it is necessary to interpret its impact on the efficacy of immunotherapy and the predictive value of drug resistance in clinical practice.

Reduced TMB and neoantigens

TMB is the number of substitution, insertion, and deletion mutations per megabase in the exon coding region of the evaluated gene. Yarchoan *et al.*[23] analyzed the TMB of 27 tumor types and found a superior ORR of immune checkpoint monotherapy for tumor types with high TMB. NSCLC and melanoma have relatively higher mutation loads and immunogenicity, and they are more sensitive to ICI therapy. On the contrary, pancreatic cancer, prostate cancer, and thyroid carcinoma have lower mutation loads and exhibit

lower immune responses[23-25]. Tumor neoantigens refer to antigens that are not expressed in normal tissues but only exist in tumor tissues, which are usually highly immunogenic. Several studies have shown that increased TMB, DNA mismatch repair gene deletion, and high genomic microsatellite instability can increase tumor antigen expression and improve immune efficacy[24,26]. Anagnostou *et al.*[27] found that the neoantigen load of NSCLC patients treated with pembrolizumab with sustained clinical benefit was significantly higher than that of patients with non-sustained benefit. The study also found that 7-18 mutation-related neoantigens that can produce anti-cancer responses were lost and replaced by more complicated new mutations before drug resistance. The proportion of new mutations involved in encoding tumor antigens had decreased (8% *vs.* 19%) and the clonality of T cell receptors (TCR) was changed, leading to the acquired drug resistance.

Decreased tumor antigen presentation

The major histocompatibility complex (MHC) is a key molecule involved in the immune response, participating in the processing and presentation of antigens, thereby activating T cells and mediating the immune response. β2-microglobulin (β2-GM) is one of the important proteins that compose the heavy chain of MHC class I molecules, which is responsible for folding and transporting MHC class I molecules to the cell surface[28]. Studies confirmed that *B2M* gene mutations can lead to the loss of MHC class I molecules on the cell surface, which in turn promotes the recognition dysfunction of CD8+ T cells and induces immune resistance in NSCLC[29]. In addition, studies found that defective expression of MHC class II molecules in tumor cells and infiltrating lymphocytes cannot effectively activate T helper cells. The low antigen presentation reduces the immunogenicity of lung cancer, while weak immunogenic tumors can escape the surveillance of the immune system and promote immunotherapy resistance in lung cancer[30].

Dynamic changes of PD-L1 expression

Tumor cells with high expression of PD-L1 can also mediate immune escape. PD-L1 could transmit negative signals and induce T cell apoptosis or dysfunction when combined with its receptor, thereby evading the immune system. In patients with acquired resistance, studies have found that interferon-γ (IFN-γ) secreted by both TILs and tumor antigen-specific T cells can mediate the upregulation of PD-L1 expression after tumor antigen recognition. In addition, there are many ways for tumors to upregulate the expression of PD-L1. For example, the currently known PTEN deletion[18], PI3K, or AKT mutations[31] can induce the expression of PD-L1 in tumor cells to escape from immune system. However, clinical trials including KEYNOTE-024 and KEYNOTE-208 have demonstrated that the high PD-L1 expression of tumor cells were more sensitive to immunotherapy[32,33]. Chinese Society of Clinical Oncology guidelines and The National Comprehensive Cancer Network guidelines also indicate that the expression level of PD-L1 can be used as one of the predictors of the efficacy of immunotherapy. These studies suggest that the functions of PD-L1 may be diverse, rather than simply categorized as "promoting" or "inhibiting". A recent study confirmed that deacetylation-dependent nuclear translocation of PD-L1 promotes immune escape and upregulates the immune checkpoint genes such as PD-L2 and VISTA of tumor cells, thereby mediating resistance to PD-1/PD-L1 inhibitors[34]. In addition, nuclear PD-L1 can also induce immune response-related gene expression, including type I and type II IFN signaling pathways, nuclear factor kappa-B (NF-κB) signaling pathways, and antigen presentation pathways. These results indicate the dual role of PD-L1, which participates in both drug resistance and immune response pathways.

Epigenetic regulation

Epigenetics is the study of heritable changes in gene expression caused by mechanisms other than changes in the nucleotide sequence of the gene, including DNA methylation, histone modification, nucleosome remodeling, and non-coding RNA expression. Epigenetic changes can affect the expression of immune

checkpoints, disrupt the process of antigen presentation, and inhibit the migration of T cells to the tumor microenvironment and the activation of T cells[35]. In addition, miRNA is also involved in the above process. Studies have shown that miR-214, miR-126, and miR-568 could promote the development and function of Treg cells and lead to T cell exhaustion[35].

Extrinsic mechanisms and factors of resistance

The extrinsic mechanisms and factors of resistance mainly refer to the tumor microenvironment (TME). The TME is the internal environment for the emergence and growth of tumor cells. Immunologists have tried to find ways to positively affect the immune response, such as the usage of cytokines, adoptive immunotherapy, and other treatments, but the results are not satisfactory. Recently, researchers are focusing on the balance between the "positive factors", such as immune effector cells, and "negative factors", such as Treg cells and MDSC in TME to enhance the efficacy of immunotherapy.

Immune effector cells

The lower infiltration and exhaustion of immune effector cells lead to immune suppression and mediate immune escape. Based on the distribution of CD8+ T cells, the immune phenotypes are divided into three types: immune inflamed, immune excluded, and immune desert. In a phase II clinical trial of durvalumab combined with olaparib in SCLC, 14 evaluable patients were included, of whom nine were immune excluded, three were immune-inflamed, and two were immune desert type. They study found that immunotherapy may play a role in the TME infiltrated by CD8+ T cells[36]. Guo *et al.*[37] analyzed the single-cell sequencing of 12,346 T cells in the peripheral blood, cancer tissue, and adjacent tissues from 14 patients with NSCLC, and they found that infiltrating T cells were mainly comprised of three subgroups. In addition to tumor-infiltrating CD8+ T cells undergoing exhaustion, two other clusters of cells exhibited states preceding exhaustion, and a high ratio of "pre-exhausted" to exhausted T cells was associated with better prognosis of lung adenocarcinoma. NK cells are not restricted by MHC or antibodies and can directly release perforin and TNF to exert their immune functions. The dysfunction of NK cells could mediate immune escape through such mechanisms as overexpression of inhibitory receptors and impaired production of cytokines. Trefny *et al.*[38] analyzed 35 patients with NSCLC who were treated with nivolumab and found a correlation between immunoglobulin-like receptor gene *KIR3DS1* expressed by NK cells and therapeutic efficacy. Mutations of *KIR3DS1* gene could lead to resistance to immunotherapy. In addition, several studies have revealed the relationship between the immune response of B cells and tertiary lymphoid structures (TLS). Compared with non-responders, responders have significantly higher B cell-related gene expression levels before ICI treatment. The density of CD20-positive B cells and TLS in the tissues and the ratio of TLS to tumor area in patients who respond to immunotherapy were significantly higher[39-41]. In general, these studies have shown the role of immune effector cells in regulating anti-tumor immunity and immunotherapy efficacy.

Treg cells

Treg cells are a type of T cell group with immunosuppressive function, which can inhibit the activation and proliferation of CD4+ and CD8+ T cells and the functions of naive and memory T cells. Treg cells play an indispensable role in maintaining the balance of autoimmunity and can effectively weaken the immunity of autoantigens. However, they are also "utilized" by tumors cells that could express autoantigens to avoid immune surveillance[42]. Elevated Treg levels were found in lung, breast, and pancreatic cancers[43], while the Treg levels were significantly reduced after surgical removal of the tumor[44]. Animal experiments[45] have proved that the depletion of CD25+ Treg cells could effectively improve the anti-tumor immune response of mice. Thus, it is believed that the elimination of Treg cells should be an essential part of the treatment. Several chemotherapeutic drugs are known to interfere with the function of Treg cells non-specifically, including cyclophosphamide, gemcitabine, methotrexate, and thalidomide[46]. Drugs for Tregs targeting

CD25 and CTLA-4 and eliminating tumor-related Treg cells include the well-known CTLA-4 antibodies ipilimumab and tremelimumab. In addition, the anti-CD25 antibody daclizumab[47], the anti-OX40 (CD134) monoclonal antibody[48], and the chemokine receptor 8[49] are in phase I clinical trials, which bring hopes for the treatment of cancer patients.

MDSCs

MDSC is a type of cell population with immunosuppressive function, which can mediate immune escape and immune tolerance through a variety of ways[50]. In addition to secreting molecules such as TGF-β and IL-10 that directly inhibit T effector cells, MDSCs can also induce the proliferation of other immunosuppressive cells. Studies have shown that MDSCs could promote the production of FOXP3+ Treg cells by secreting IFN-γ and IL-10[51,52]. MDSCs can also exert immunosuppressive effects by blocking lymphocyte homing and regulating enzymes required for adenosine metabolism. Kim *et al.*[53] found that the MDSC level of tumor-bearing mice that are resistant to ICIs increased by 5-7 times compared with non-tumor-bearing mice. They also found that a high level of PI3Kγ in MDSCs can promote the production of inflammatory mediators and immunosuppressive factors. When combining PI3K inhibitors with PD-1 /CTLA-4 inhibitors, the MDSC level of tumor-bearing mice decreased significantly and was comparable to the control group. De Henau *et al.*[54] verified that PI3Kγ inhibitors could remodel the immune microenvironment and prevent tumor growth. High expression of indoleamine 2,3-dioxygenase (IDO) was found on MDSCs in immune-resistant lung cancers. Previous studies showed that the expression level of IDO was positively correlated with clinical stages[55]. The application of IDO inhibitor INCB023843 downregulates the expression of IDO and increases the infiltration of CD8+ T cells, thereby reactivating the T cell anti-tumor response[56]. These studies reveal the potential value of suppressing MDSCs and its combination with immune checkpoint inhibitors.

TAMs

Circulating monocytes and macrophages are recruited into the tumor site and change the tumor microenvironment in the process of tumor progression. The phenotypes of macrophages could be transformed with the changes of signals produced by tumor and stromal cells. Macrophages could be divided into two categories based on their functions. M1 type macrophages are related to inflammatory response, pathogen elimination, and anti-tumor immunity, while M2 type macrophages have tumor-promoting properties. TAMs are very similar to M2 type macrophages[57]. Studies of multiple tumor types including breast cancer[58], ovarian cancer[59], and NSCLC[60] have shown that the aggregation of macrophages is positively correlated with CCL2 levels. In addition to CCL2, other chemokines such as CCL3, CCL4, CCL5, and cytokines are also involved in the recruitment of macrophages[61,62]. TAMs inhibit the function of T cells by weakening the ability of antigen presentation and releasing immunosuppressive factors such as IL-10 and TGF-β. Studies have shown that the inhibition of the recruitment of macrophages through the regulation of chemotactic agents is effective. For example, bindarit is used to inhibit CCL2[63], and monoclonal antibodies that block VEGFR2 reduce macrophage infiltration and tumor growth[64]. CSF-1R blockade can also reduce the number of TAMs and activate T cells in TME[65]. These preclinical trials have provided a basis for reversing the drug resistance raised by TAMs.

Metabolic changes in the TME

Metabolic changes in the TME can reduce immune effects by producing immunosuppressive metabolites to inhibit immune cell infiltration[66]. For example, some tumors use glutamine as an energy source, and the metabolically decomposed ammonia can activate autophagy in neighboring immune cells. Arginine metabolism plays an important role in the activation of T cells and the regulation of immune response. The immunoregulatory cells expressing arginase 1 (ARG1) in TME degrade arginine and restrict its use by T

cells. Therefore, inhibition of arginase in TME can enhance the efficacy of ICIs. A clinical trial of the combination of ARG1 inhibitor INCB001158 and nivolumab is ongoing. Besides, the rate of fatty acid synthesis is increased to produce cell membrane phospholipids and signal molecules in tumor cells, and targeting these metabolic pathways is a promising way to enhance anti-tumor immunity. In recent years, many studies have focused on the metabolic process of the TME, providing new perspectives for overcoming immune resistance.

Host-related factors

The immune system function degenerates with age, and the number and function of antigen-presenting cells and lymphocytes are also decreased[67]. A study found that elderly patients with melanoma responded better to immunotherapy than younger people[68]. A meta-analysis including 11,351 cases with advanced or metastatic cancer found that male patients had superior efficacy of immunotherapy[69]. Compared with the control group, the overall survival HR of male and female patients treated with ICIs was 0.72 and 0.86, respectively (P = 0.0019). In addition, studies have suggested that weight can also affect the efficacy of immunotherapy. A study conducted a pooled analysis of four studies of OAK, POPLAR, BIRCH, and FIR, reporting a correlation between the overall survival of atezolizumab in patients with advanced NSCLC and body mass index[70]. The results show that obesity was associated with a significant increase in the OS of patients receiving PD-L1 inhibitors treatment, and the risk of death in obese patients was reduced by 64% (HR = 0.36, 95%CI: 0.21-0.62). Smoking is also a crucial factor. The average frequency of gene mutations in smokers is more than 10 times higher than that of non-smokers[71]. A recent study reported that benzopyrene in cigarettes can induce an increase in PD-L1 level, which further explains why smoking patients respond better to immunotherapy[72]. In addition, studies have shown that gut microbiota have a significant impact on the efficacy of immunotherapy[73]. There is evidence of the importance of the intestinal microbiota in the response to chemotherapy and immunotherapy and how their alteration and the concomitant use of antibiotics inhibit the benefit of ICIs in advanced cancer, decreasing OS and PFS in NSCLC. With respect to microbiota composition, the relative abundance of *Akkermansia muciniphila* appears to significantly affect the clinical response to anti-PD-1/PD-L1 therapy in NSCLC and renal cell carcinoma[74]. Another study demonstrated that a greater diversity of the gut microbiome is related to favorable responses to ICI therapy in NSCLC[75]. Among the different immune cells, the microbiota have been shown to be associated with the development of effector cells of the immune system, such as Th1, Th2, Th17, and Treg cells[76-78]. However, in the case of SCLC, there is little evidence that supports the role of the microbiome as an immune resistance mechanism. Thus, host-related factors could also affect the efficacy of immunotherapy.

IMMUNOTHERAPY-BASED COMBINATIONS TO COPE WITH RESISTANCE

There are no standard treatments after resistance of immunotherapy. At present, the most promising and effective treatment methods are immunotherapy combinations that aim to convert "cold" tumors with lower immune response into "hot" tumors with better response. A variety of combined treatment strategies, such as immunotherapy combined with targeted therapy, chemotherapy, and radiotherapy can modulate the immune response at different stages and overcome drug resistance. Clinical trials of immunotherapy-based combinations related to lung cancer patients are summarized in Table 2.

Dual immunotherapy

Anti-tumor immune response involves antigen recognition, presentation, and activation of immune cells. Currently, many studies focus on the immune checkpoint inhibitors targeting PD-1, PD-L1, lymphocyte activating gene 3 (LAG-3), T cell immunoglobulin domain and mucin domain-3 (TIM-3), and T cell immunoglobulin and ITIM domain protein (TIGIT). The combined application of these immune

Table 2. Clinical trials of immunotherapy-based combinations related to lung cancer patients

Immunotherapy-based combinations	Combination strategies	Drugs	Clinical trials
Dual immunotherapy	Anti-PD-1 + Anti-CTLA-4	Nivolumab + Ipilimumab	Checkmate 227 (NCT02477826)
	Anti-PD-1 + Anti-LAG-3	Nivolumab + MK-4280	Keynote 495 (NCT03516981)
		Nivolumab + Relatimab	NCT03607890
		Pembrolizumab + IMP321	NCT03625323
	Anti-PD-1/PD-L1 + Anti-TIGIT	Atezolizumab + Tiragolumab	CITYSCAPE (NCT03563716)
		Pembrolizumab + MK-7684	NCT02964013
	Anti-PD-L1 + Anti-TIM-3	LY3300054 + LY3321367	NCT03099109
ICIs combined with CT	Anti-PD-1/PD-L1 + CT	Pembrolizumab + Pemetrexed/Platinum	Keynote 189 (NCT02578680)
			Keynote 021 (NCT02039674)
		Atezolizumab + Paclitaxel/Carboplatin	IMpower 130 (NCT02367781)
			IMpower 131 (NCT02266949)
		Atezolizumab + Etoposide/Carboplatin	IMpower 133 (NCT02763579)
ICIs combined with targeted therapy	Anti-PD-L1 + Anti-VEGF + CT	Atezolizumab + Bevacizumab + Paclitaxel/Carboplatin	IMpower 150 (NCT02366143)
	Anti-PD-L1 + Anti-RTK	Nivolumab + Sitravatinib	NCT02954991
	Anti-PD-L1 + Multitarget inhibitor	Atezolizumab + Cabozantinib	COSMIC-021 (NCT03170960)
ICIs combined with RT	Durvalumab after concurrent RT + CT		PACIFIC (NCT02125461)
	Pembrolizumab + SBRT		PEMBRO-RT (NCT02492568)
Individualized immunotherapy	Anti-PD-1 + Vaccine	Nivolumab + NEO-PV-01	NCT02897765
	Anti-PD-1 + MDM2 inhibitor	Pembrolizumab + APG-115	NCT03611868

ICIs: Immune checkpoint inhibitors; PD-1: programmed cell death 1; PD-L1: programmed cell death ligand 1; CTLA-4: cytotoxic T lymphocyte-associated antigen-4; LAG-3: lymphocyte activation gene-3; TIGIT: T cell immunoglobulin and ITIM domains; TIM-3: T cell immunoglobulin-3; CT: chemotherapy; RTK: receptor tyrosine kinases; RT: radiotherapy; SBRT: stereotactic body radiation therapy; MDM2: mouse double minute 2 homolog.

checkpoint inhibitors could exert synergistic effects. The most common strategy is PD-1 inhibitor combined with anti-CTLA-4 antibody. Both CheckMate 227 and CheckMate 032 studies have confirmed that the efficacy of dual-immunotherapy is better than that of single-agent therapy[79,80]. LAG-3 is expressed on the activated CD4+, CD8+ T cells, NK cells, and Treg cells, which can bind to its ligand fibrinogen-like protein-1 (FGL1) to inhibit T cell function. The trial of LAG-3 antibody MK-4280 combined with pembrolizumab in advanced NSCLC (KEYNOTE-495/NCT03516981) recruited 33 patients with solid tumors who have failed previous treatments. The DCR of the monotherapy group and the combination treatment group was 17% and 40%, respectively. TIGIT is a specific negative regulator of CD226 costimulatory receptor and plays an important role in immunosuppression. The CITYSCAPE study enrolled 135 patients with PD-L1-positive treatment-naïve NSCLC who were randomly assigned to TIGIT antibody tiragolumab combined with atezolizumab (TA group) or placebo combined with atezolizumab (PA group). The results show that, in the intention-to-treat population, the ORR and PFS of the TA group was increased compared with the PA group (31.3% *vs.* 16.2%; 5.4 months *vs.* 3.6 months), and the risk of disease progression was reduced by 43%[81]. In addition, the phase II clinical trial of relatimab combined with nivolumab in the treatment of solid tumors (NCT03607890) and the phase II clinical trial of IMP321

combined with pembrolizumab in the treatment of patients with advanced NSCLC (NCT03625323) are both underway. The phase I clinical trial of pembrolizumab combined with TIGIT antibody in the treatment of patients with metastatic solid tumors (NCT02964013) and the phase I trial of TIM-3 inhibitor combined with PD-L1 antibody in the treatment of advanced relapsed/refractory solid tumors (NCT03099109) are ongoing.

Immunotherapy combined with chemotherapy

Immunotherapy combined with chemotherapy can not only increase the cross-presentation of antigens by DCs[82] but also eliminate the immunosuppressive components of the TME[83], such as Treg cells, MDSCs, and immunosuppressive cytokines. The KEYNOTE-189 study showed that the efficacy of pembrolizumab combined with chemotherapy in patients with advanced NSCLC in first line is superior to chemotherapy alone[84]. The OS of the two groups was 22.0 and 10.7 months and the PFS was 9.0 and 4.9 months, respectively. In addition, the CheckMate 012, KEYNOTE-021, IMpower130, and IMpower131 studies have shown that immunotherapy combined with chemotherapy has a greater survival benefit than chemotherapy along despite using different checkpoint inhibitors. Based on these studies, the U.S. Food and Drug Administration has approved nivolumab and pembrolizumab combined with platinum-based chemotherapy for EGFR/ALK wild-type NSCLC. Besides, results from the double-blind, randomized phase III study IMpower133 show that, compared with chemotherapy alone, atezolizumab combined with chemotherapy in the first-line treatment of extensive-stage SCLC achieved OS benefit, which represents a breakthrough in the treatment of SCLC[85].

Immunotherapy combined with targeted therapy

Targeted therapy can increase tumor antigens and play a synergistic effect with immunotherapy in multiple aspects. Anti-angiogenic agents can promote the normalization of blood vessels, enhance immune effector cells to infiltrate the tumor, and reduce the activity of immunosuppressive cells. The IMpower150 study evaluated atezolizumab combined with bevacizumab and chemotherapy in the first-line treatment of non-squamous NSCLC patients, including EGFR and ALK mutant populations, and the results confirm that patients in ABCP group have significant survival benefits[86]. Cohort 7 of the COSMIC-021 study included 30 advanced NSCLC patients with negative driver genes who had previously been treated with immune checkpoint inhibitors. The results show that the ORR was 23%, DCR was 83%, and the median duration of response was 5.6 months[87]. A phase I randomized clinical study explored the efficacy of nivolumab combined with erlotinib in the treatment of patients with EGFR-mutant advanced NSCLC. Twenty EGFR-TKI resistant cases and one EGFR-naive case were included. The results show that the median PFS was 5.1 months[88]. In addition, the combination of novel targeted agents with immunotherapy has also shown great prospects. Nivolumab combined with the small molecule inhibitor sitravatinib in the treatment of patients with advanced non-squamous NSCLC who are resistant to immunotherapy obtained an ORR of 32.14%, a median PFS of 6.8 months, and a median OS of 15.1 months[89]. Pembrolizumab combined with JAK1 inhibitor itacitinib in the treatment of NSCLC patients with PD-L1 expression > 50% reported an ORR of 66.7%.

Immunotherapy combined with radiotherapy

The PACIFIC study included 713 patients with locally advanced unresectable NSCLC. The results show that the median PFS of the durvalumab treatment group after concurrent radiotherapy and chemotherapy was 17.2 months, and the median OS was also significantly improved compared with control group[90]. The PEMBRO-RT study explored the efficacy of radiotherapy and concurrent immunotherapy. Patients were treated with pembrolizumab seven days after receiving stereotactic body radiotherapy, while the control group only used immunotherapy. The ORR of the two groups was 36% and 18% and the median OS was 15.9 and 7.6 months, respectively[91]. In addition, a retrospective study included 26 NSCLC patients with

acquired resistance of immunotherapy. In total, 15 patients were administered with local treatment without systemic treatment, and 11 of them continued to receive immunotherapy after local treatment. The two-year survival rate was 92%, and the median OS has not yet been reached[92]. Similarly, a retrospective study including 189 patients with acquired resistance showed that local treatment can significantly improve survival benefits[93].

Other combination strategies are also being explored in immunotherapy-resistant populations. The VARGADO study included 57 patients with advanced NSCLC after progression with second-line immunotherapy. Patients were treated with the VEGF inhibitor nintedanib combined with docetaxel. The results show that ORR and DCR are 50% and 85%, respectively. The median PFS was 6.5 months and the median OS was 12.4 months[94]. Although the ideas for overcoming immunotherapy resistance are various, there exist some problems that cannot be ignored while conducting clinical trials. Firstly, adequate pre-clinical evaluation should be carried out when the clinical trial is designed, and the adverse effects should be monitored. For example, in the PACIFIC study, the incidence of radiation pneumonitis in the immunotherapy group was increased (33.9% *vs.* 24.8%), and 15.4% of patients discontinued treatment due to adverse events. In the TATTON study, the incidence of interstitial pneumonitis in NSCLC patients receiving durvalumab combined with osimertinib was significantly increased; thus, the treatment group was promptly interrupted in the corresponding phase III clinical trial (CAURAL study). Novel treatment strategies such as immunotherapy combined with molecular inhibitors need to further consider the potential safety profiles raised by the superposition of different drugs. Secondly, the rationality of the combination strategy is supposed to be evaluated. The selected treatment drugs and immune checkpoint inhibitors should have synergistic effects in the immune feedback loop.

Individualized immunotherapy

Given the characteristics of the tumor immune microenvironment, developing drugs for tumor cell neoantigen production and presentation, T cell activation, and immunosuppressive microenvironment, such as oncolytic viruses, tumor vaccines, and adoptive immune cell therapy (TIL, CAR-T, TCR-T, CAR-NK, *etc.*), which are based on the characteristics of individualized immune microenvironments represent promising directions for overcoming immunotherapy resistance. The NT-001 study is a phase Ib clinical trial that intends to explore the efficacy of PD-1 inhibitors combined with vaccines for advanced or metastatic melanoma, smoking-related NSCLC, and bladder cancer. In total, 82 patients were enrolled. ORR for metastatic NSCLC patients reached 45.5%. MDM2 (mouse double minute 2 homolog) is one of the most important inhibitors of p53; it degrades the p53 protein and weakens its tumor suppressor effect. APG-115 is a second-generation MDM2 inhibitor that can block the MDM2-p53 interaction, thereby restoring the transcriptional regulatory function of the p53 protein, promoting cell apoptosis, and restoring tumor suppressor activity. The phase Ib clinical trial results of APG-115 combined with pembrolizumab in the treatment of metastatic melanoma or advanced solid tumors confirm that the combination of the two drugs is well tolerated, with an ORR of 16.7% and a DCR of 55.5%. With the continuous in-depth study of immunotherapy mechanisms, individualized immunotherapy targets may be a new strategy with good development prospects.

CONCLUSIONS AND FUTURE DIRECTIONS

Compared with traditional chemotherapy and targeted therapy, immunotherapy has its unique advantages and brings a new light to lung cancer treatment. With the clinical application of immunotherapy, anti-PD-1 /PD-L1 monotherapy resistance has become an unavoidable phenomenon. The ongoing research on the mechanisms of immune resistance provides new ideas for the selection of patient populations and response strategies for reversing immune resistance. Immunotherapy-based combinations are becoming one of the

most promising strategies that convert non-responders to responders. In the future, studies on biomarker identifications are required to predict the efficacy and prognosis of immunotherapy. Further exploration is needed to predict immune resistance and the timing of restarting immunotherapy.

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Authors' contributions

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Manuscript revision: Su C
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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

Review

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Resistance to immune checkpoint inhibitors in KRAS-mutant non-small cell lung cancer

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Abstract

Non-small cell lung cancer (NSCLC) patients with Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation are associated with significant clinical heterogeneity and a poor prognosis to standard NSCLC therapies such as surgical resection, radiotherapy, chemotherapies, and targeted medicines. However, the application of immune checkpoints inhibitors (ICIs) has dramatically altered the therapeutic pattern of NSCLC management. Clinical studies have indicated that some KRAS-mutant NSCLC patients could benefit from ICIs; however, the responses in some patients are still poor. This review intends to elucidate the mechanisms of resistance to immunotherapy in KRAS-driven NSCLC and highlight the TME functions altered by immunoinhibitors, immunostimulators, and cancer metabolism. These metabolic pathways could potentially be promising approaches to overcome immunotherapy resistance.

Keywords: Non-small cell lung cancer, KRAS mutation, immune checkpoints inhibitors, tumor microenvironments, cancer metabolism.

INTRODUCTION

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths worldwide[1]. According to Global Cancer Statistics 2020, lung cancer is estimated to represent 11.4% of new cancer cases and 18.0% of cancer-related deaths[1]. There are two main histological types of lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[2]. About 85% of lung cancer is NSCLC, which comprises lung adenocarcinoma (LUAD), lung squamous cell carcinoma, and large cell neuroendocrine carcinoma. SCLC accounts for the remaining 15%[3]. Despite inducements such as smoking, genetic mutations are the leading causes of lung cancer. The most common driver mutations in NSCLC involve Kirsten rat sarcoma viral oncogene homolog (KRAS), epidermal growth factor receptor (EGFR), and anaplastic lymphoma kinase[4].

KRAS, a member of the RAS family, was one of the first oncogenes identified in NSCLC[5]. Since KRAS mutation is reported to be associated with resistance to multiple therapies and poor prognosis in NSCLC, several preclinical and clinical studies have investigated effective therapies, including immunotherapy and targeted therapy[6,7]. In 2021, AMG510, specifically targeting $KRAS^{G12C}$, was approved by the FDA as the orphan drug to treat the NSCLC with $KRAS^{G12C}$[8]. Other inhibitors, peptides, and tumor vaccines are under preclinical and clinical studies, including MRTX849 targeting $KRAS^{G12C}$, MRTX1133 targeting $KRAS^{G12D}$, 12VC1 targeting $KRAS^{G12C/V}$, mRNA-5671 targeting $KRAS^{G12C/D/V}$, *etc.*[9]. In addition to targeted therapy, immunotherapies have also remarkably changed the management of NSCLC[10]. The increased expression of programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) has also been demonstrated to be closely related to KRAS status[11]. Controversial results have been reported in multiple clinical studies on the efficacy of immune checkpoints inhibitors (ICIs) in NSCLC with KRAS mutation[12-15]. Jeanson *et al.*[16] and Arbour *et al.*[13] demonstrated no difference in response to ICIs among NSCLC patients with or without KRAS mutations. However, other studies have demonstrated more benefits from ICIs for NSCLC patients with KRAS mutation than those without[14,15]. In this review, we summarize and analyze the possible mechanisms underlying the resistance to ICIs in NSCLC with KRAS mutation and mainly focus on the role of the tumor microenvironment (TME) and metabolism, therapeutic implications, and potential targets for overcoming the resistance to or improving the efficacy of ICIs treatments in KRAS-mutant NSCLC.

ICIS TREATMENTS IN NSCLC WITH KRAS MUTATION

Even though the status of KRAS mutation as being able to alter the responses to ICIs in NSCLC has been verified in multiple fundamental studies, solid clinical evidence is still lacking. In this section, we review the clinical trials of ICIs in KRAS-mutant NSCLC patients.

Checkmate-057 is a random and double-blind phase 3 clinical trial with a size of 582 cases, which is designed to compare the efficacy of monotherapy of nivolumab or docetaxel in advanced non-squamous NSCLC patients. Comparing to docetaxel, nivolumab significantly elongates the overall survival (OS) (medium OS = 12.2 months *vs.* 9.4 months; HR = 0.73; 95%CI: 0.59-0.89; $P = 0.0002$). The subgroup analysis showed that KRAS-mutant patients have better survival benefits than KRAS-wildtype patients (HR = 0.52, 95%CI: 0.29-0.95; HR = 0.98, 95%CI: 0.29-0.95)[17]. Another phase 3 clinical trial, KEYNOTE-042, investigated the efficacy and safety of monotherapy of pembrolizumab or platinum-based chemotherapy in locally advanced or metastatic NSCLC harboring wildtype EGFR/anaplastic lymphoma kinase (ALK) and PD-$L1^{+}$ (TPS ≥ 1%). The results show that the monotherapy of pembrolizumab can prolong the OS compared to platinum-based chemotherapy. Further analysis revealed that KRAS-mutant patients show higher PD-L1 expression and tumor mutational burden (TMB) and have significantly longer progression-free survival (PFS) in the pembrolizumab treatment group than those in the platinum treatment group[14,18].

KEYNOTE-189 evaluated the efficacy and safety of pemetrexed and platinum-based therapy or combined with pembrolizumab in metastatic non-squamous NSCLC patients. The results demonstrate that a combination of pembrolizumab and chemotherapy can significantly prolong the OS than chemotherapy regardless of the PD-L1 expression level. Both KRAS-wildtype and -mutant NSCLC patients benefit from chemotherapy combined with pembrolizumab. The pooled analysis of KEYNOTE-189 and KEYNOTE-042 indicated that the non-squamous NSCLC patients with wildtype EGFR/ALK or KRAS show a better prognosis to the combination of pembrolizumab and chemotherapy (HR = 0.55) compared to pembrolizumab monotherapy (HR = 0.86). However, for KRAS-mutant non-squamous patients, the monotherapy of pembrolizumab shows a better prognosis (HR = 0.42 and HR = 0.28) than combination treatment of pembrolizumab and chemotherapy (HR = 0.79 and HR = 1.14)[19]. Altogether, the clinical outcomes above indicate that KRAS-mutant NSCLC patients can benefit from immunotherapy or combined treatment of immunotherapy and chemotherapy, whereas the conclusion is not supported by some meta-analyses with more solid evidence. Two meta-analyses and some retrospective analyses have reported that the status of KRAS mutation has no negative association with the survival outcome of immunotherapy in advanced NSCLC patients[7,16,20,21].

One possible illustration is that the co-mutations besides KRAS mutation might play the predictive and prognostic role in response to ICIs in KRAS-mutant NSCLC. Skoulidis *et al.*[22] defined three distinct co-mutation subgroups of the early stage and advanced KRAS-mutant tumors: $KRAS^{mut}$/serine/threonine kinase 11 $(STK11)^{-/-}$, $KRAS^{mut}$/tumor protein 53 $(TP53)^{-/-}$ (KP), and $KRAS^{mut}$/cyclin dependent kinase inhibitor 2A, 2B $(CDKN2A, 2B)^{-/-}$/thyroid transcription factor 1^{low} (KC)[22]. The retrospective analysis revealed that the KL patients show less responses to immunotherapy and shorter PFS and OS than KP patients[23]. Other investigations have revealed that KP patients show significant benefits from PD-1 blockade monotherapy[24]. Collectively, PD-L1 blockade might be more beneficial to KP patients than KL patients.

Taken together, not all the KRAS-mutant NSCLC patients could benefit from ICIs. Co-mutations increase the TMB in patients and might contribute to the poor response to ICIs. The mechanisms of resistance to ICIs in NSCLC with KRAS mutation are sophisticated and not fully investigated, which we discuss in the following sections.

THE RESISTANT MECHANISMS OF ICIS TREATMENTS IN NSCLC WITH KRAS MUTATION

KRAS regulates TME through immunomodulatory molecules

Growing evidence has implicated that the intrinsic and extrinsic resistance mechanisms to ICIs might result from the immunosuppressive TME caused by alterations in disparate signaling pathways in tumor cells[25]. Previous studies have clearly indicated that the immunosuppressive TME can be regulated by alterations of immunoinhibitors and immunostimulators in tumor and stromal cells[26].

Immune checkpoints exert crucial roles in preventing overreaction and minimize the duration and expansion of immune responses[27]. PD-1 (CD279), coded by *pdcd1*, is ubiquitously expressed on various immune cells, including activated T cells, B cells, monocytes, NK cells, and dendritic cells (DCs)[28]. PD-1 can be recognized by the ligands, PD-L1 and PD-L2, on normal tissue cells and cancer cells, which maintains the immune homeostasis but might also revoke the anti-tumor immunity[29]. After PD-1 binds to PD-L1, PD-1 undergoes a conformational change, which leads to the inactivation of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3K)-serine/threonine kinase (AKT) and extracellular signal-regulated kinase 2 (ERK) pathways and, consequently, dysfunction of T cells[30]. In addition, studies have reported that tumor cells can secrete exosomes carrying PD-L1, which impede the function of $CD8^+$ T

cells, $CD4^+$ T cells, and Tregs before infiltration[31]. It has been widely reported that oncogenic KRAS can increase PD-L1 from different aspects, including transcription, stabilization, and recycling. In LUAD cell lines, $KRAS^{mut}$ has been found to increase the transcriptional level of PD-L1 through the mitogen-activated protein kinase kinase 1 (MEK) pathway[32]. In human lung and colorectal cancer, Coelho *et al.*[33] demonstrated that the adenylate-uridylate-rich element-binding protein tristetraprolin (TTP) can bind to the 3' untranslated region of PD-L1 mRNA and consequently induce the degradation of PD-L1. The oncogenic KRAS activates MEK signaling and further phosphorylates and inhibits the TTP by MK2, which leads to the stabilization of PD-L1 mRNA[33]. In addition, it has been reported that mutant KRAS suppresses programmed cell death 4 and then promotes the translation of ADP ribosylation factor 6 and MYCBP associated protein expression, which facilitates PD-L1 recycling driven by platelet-derived growth factor and eventually the immune evasion[34].

As another factor, cytokine-modulated immune responses have been investigated for decades. Cytokines are a type of small proteins secreted by almost all types of cells and can be classified into seven families: interleukin (IL), colony stimulating factor (CSF), interferon (IFN), tumor necrosis factor, tumor growth factor-beta (TGF-β) family, growth factor (GF), and chemokine family[35]. Cytokines deliver messages in a paracrine, autocrine, or endocrine manner and can coordinate the recruitment of immune cells, spatial organization, and cellular interactions[36,37]. Plentiful cytokines and chemokines have both pro- and anti-inflammatory functions and exert different functions in particular scenarios[36]. It has been reported that mutant KRAS can remodel the TME by regulating the production and secretion of pro- or anti-inflammatory cytokines and chemokines[38]. The oncogenic mutant KRAS represses the expression of interferon regulatory factor 2 (IRF2) and results in increased production of C-X-C motif chemokine ligand 3 (CXCL3), which binds to C-X-C motif chemokine receptor 2 (CXCR2) on myeloid-derived suppressor cells (MDSCs) and subsequently recruits the MDSCs to impede the cytotoxic T cells[39]. In lung cancer, the ubiquitin-specific protease 12 (USP12) induces protein phosphatase 1B, which thwarts transcription factor P65 (NF-κB). NF-κB can promote the production of multiple chemokines, including CXCL8, CXCL1, CCL2, *etc.*, which can result in an immunosuppressive TME through recruiting the M2 macrophages, inducing PD-L1 expression and blocking the responses to T cells. Oncogenic mutant KRAS inhibits USP12 and further promotes the production of chemokines through NF-κB, which ultimately causes the resistance to anti-PD-1[40]. Additionally, it has been reported that the development of resistance to anti-PD-L1 and MEK inhibition is the result of increasing IL-17 and IL-22 secreted by accumulated infiltration of Th17 in $KRAS^{mut}/TP53^{-/-}$ co-mutant lung cancer patients[41]. Moreover, KRAS has been reported to induce the releasing of pro-inflammatory cytokines (IL-6, IL-8, and IL-1) and anti-inflammatory cytokines (IL-10, IL-22, and TGF-β), as reviewed by Hamarsheh *et al.*[42].

Immunomodulators are composed of all molecules that can regulate the immune response, including receptors/ligands on both tumor cells and stromal cells in TME and cytokines and chemokines secreted by cells. The studies cited above have uncovered that KRAS could mediate the rearrangement of immunomodulators through different signaling pathway in multiple cancers, which might contribute to the resistance to ICIs in NSCLC. A schematic figure of KRAS-driven signaling pathways in the regulation of immunomodulators is presented in Figure 1.

KRAS regulates TME through metabolic alteration

For the past decades, many studies have revealed adjustments of energy metabolism in cancers, which are involved in almost every stage of cancer development[43,44]. Oncogenic KRAS mutation has been reported to regulate diverse metabolic networks to fulfill the excessive requirement of distinct nutrients to support tumor growth and metastasis[45,46]. In this section, we discuss the mechanisms underlying how KRAS orchestrates the TME through metabolic networks.

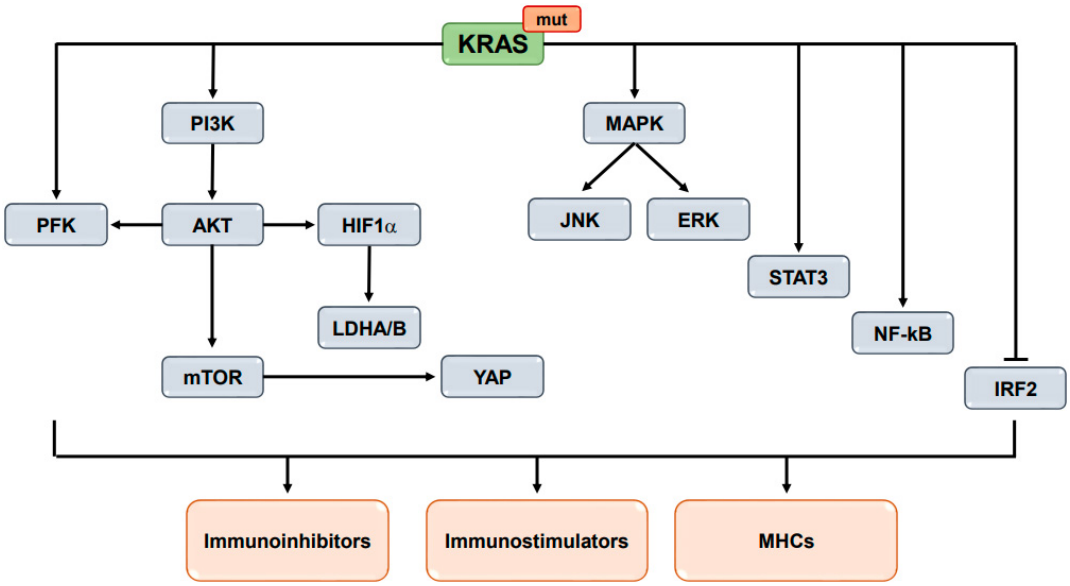

Figure 1. A schematic figure of KRAS-driven signaling pathways in the regulation of immunomodulators. PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; AKT: serine/threonine kinase; MAPK: mitogen-activated protein kinase 1; ERK: extracellular signal-regulated kinase 2; PFK: phosphofructokinase; HIF1α: hypoxia inducible factor 1 subunit alpha; JNK: mitogen-activated protein kinase 8; mTOR: mechanistic target of rapamycin kinase; LDHA/B: lactate dehydrogenase A/B; YAP: yes1 associated transcriptional regulator; IRF2: interferon regulatory factor 2; STAT3: signal transducer and activator of transcription 3; NF-κB: transcription factor P65; MHCs: major histocompatibility complex.

Glycolysis

In normal cells, glucose can be consumed either through oxidative phosphorylation (in mitochondria) or anaerobic glycolysis (in cytosol) to produce energy for cell activities. Glucose is mainly transported into cells with the help of glucose transporters (GLUTs) in an energy-consuming-free way. In cytosol, glucose is catalyzed to glucose-6-phosphate (G6P) by hexokinase (HK). G6P can enter the pentose-phosphorylation pathway with catalyzation of G6P dehydrogenase and be processed to ribulose-5-phosphate for the synthesis of purine. G6P can also be isomerized to fructose-6-phosphate (F6P) and further phosphorylated to fructose-1,6-biphosphate (FBP), which enters the glycolysis pathway. Under the aerobic condition, FBP is catalyzed to pyruvate and then converted to acetyl-CoA by pyruvate dehydrogenase (PDH), which participates in the TCA cycle. Under the anaerobic condition, pyruvate does not enter the TCA cycle but is converted to lactate by the lactate hydrogenase (LDH). Under normal condition, a small portion of F6P also enters the hexosamine pathway (HBP) with the help of glutamine--fructose-6-phosphate aminotransferase (GFPT) and provides glycans for protein glycosylation[47,48].

Back in 1985, it was reported that rat-1 cell maintains a higher level of glycolysis in the presence of *ras* transfection[49]. Accumulating evidence shows that oncogenic KRAS mutation modulates glycolysis through promoting the uptake of glucose and increasing glycolysis flux. In pancreatic ductal adenocarcinoma (PDAC) with $KRAS^{G12D}$, metabolomic and RT-qPCR analyses have revealed increasing glucose transporter solute carrier family 2-member 1 (GLUT1) expression and enhanced glycolysis flux. $KRAS^{G12D}$ was found to upregulate the expression of several enzymes, including HK, PFK, enolase, *etc.*[50]. Many studies have explored in more detail about how mutant KRAS interacts with the enzymes involved in the carbon metabolism pathways. In KRAS-driven PDAC, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 is phosphorylated by p38γ induced by KRAS mutation and forms a ternary complex with solute carrier family 2 member 2 (GLUT2), which promotes the uptake of glucose[51]. In addition, GLUT1 has been found significantly upregulated in NSCLC with KRAS mutation[52]. KRAS4A, a shorter isoform of KRAS, has been

found to bind to the N-terminal of HK1 and block the allosteric inhibition feedback, which promotes the transition of glucose to G6P and increases the glycolysis flux[53]. Based on both an *in vivo* mice model and an *in vitro* H23 cell line study, Wang *et al.*[54] reported that KRAS activation is required for HK2 expression. In a $KRAS^{mut}/STK11^{-/-}$ co-mutated lung cancer model, KRAS mutation induces the expression of GLUT. Meanwhile, the loss of STK11 impedes the phosphorylation and activation of protein kinase AMP-activated catalytic subunit alpha 1 (AMPK), thereby leading to the activation of GFPT enzyme. The increasing activity of GLUT and GFPT promotes HBP[55]. Glucose is also the primary energy source for the proliferation and differentiation of immune cells[56,57]. As a result of increasing glycolysis in tumor cells, the access to glucose for immune cells is limited in TME. A cruel competition between tumor cells stromal cells consequently impairs the glycolytic metabolism and normal functions of immune cells[58,59]. More specifically, the limited glycolysis causes the weakened activity of the AKT/mammalian target of rapamycin (mTOR) pathway in T cells, which impedes the production of INF-γ[59]. The limited availability of glucose also results in insufficient production of glycolytic metabolite phosphoenolpyruvate to maintain the anti-tumor activity through calcium and NFAT signaling pathways in infiltrated T cells[60]. In NK cells, the aberrant expression of fructose-1,6-bisphosphatase (FBP1) in gluconeogenesis and TCA cycle leads to the halt in glycolysis and induction in cell death[61]. It has also been found that inhibition of glycolysis impairs the oligomerization of C-C motif chemokine receptor 7 and thus hinders the migration of DCs to draining lymph nodes[62].

One important metabolite of glycolysis is lactate. In KRAS-driven NSCLC, the high expressions of LDH-A and LDH-B are reported to be associated with tumorigenesis and disease progression[63,64]. One study found that phosphoglycerate kinase 1 (PGK1) can be phosphorylated by ERK1/2, which is the downstream of KRAS. The phosphorylated PGK1 phosphorylates PDHK1, the latter further phosphorylating and inhibiting PDH, which blocks the conversion of pyruvate to acetyl-CoA and subsequently promotes the conversion of pyruvate to lactate[65]. In PDAC, hypoxia and active KRAS can induce the expression of hypoxia inducible factor 1 subunit alpha (HIF1α) and inhibit the degradation of HIF1α, respectively. HIF1α then transcriptionally enhances the expression of the lactate transporter, solute carrier family 16 member 4 (MCT4), which transports lactate out of cells[66]. The accumulation of lactate from the excessive glycolysis in tumor cells is transported by MCT4 out of cells, which generates a low-pH environment that discourages the normal proliferation and function of immune cells[67]. The accumulated lactate promotes the polarization of macrophages toward anti-inflammatory M2 phenotype and leads to a downregulation of interferon gamma (IFN-γ) secreted by the infiltration of T cells and NK cells[68,69]. Besides, the accumulated lactate directly blocks the glycolytic flux in T cells[70].

Taken together, the studies described above suggest that KRAS mutation can promote glycolysis by promoting various signaling pathways and molecules, such as related enzymes and transporters. The outrageous requirement of glucose and excessive excretion of lactate impair the normal proliferation and function of different immune cells, which ultimately diminishes the anti-tumor activity.

Lipid metabolism

Lipid metabolism is constantly in homeostasis due to the balance of fatty acids synthesis (FAS) and fatty acids oxidation (FAO) in normal cells. In the process of *de novo* lipogenesis, citrate is catalyzed to acetyl-CoA in assistance with ATP-citrate lyase (ACLY) and then transformed to palmitate by FA synthetase (FASN), phosphatidic acids, and finally triacylglycerol. Triacylglycerol can be stored in the lipid droplets (LDs) in the cytoplasm[71]. Phospholipids are another source of lipogenesis and can be converted to arachidonic acid (ARA) by phospholipases (PLA2)[72]. ARA can be catalyzed by cyclooxygenase 1 and 2 (COX-1 and COX-2) and transformed to prostaglandins 2 (PGE2), which has been shown to play a vital

role in tumorigenesis and immune response[73]. The FAO process can be classified into harmless β-oxidation and fatal lipid peroxidation. In β-oxidation, FAs are catalyzed to produce acetyl-CoA, NADH, and FAD. Besides, lipid peroxidation is believed to start from polyunsaturated fatty acid chains, induced by reactive oxygen species (ROS), and results in ferroptosis[74]. In the mouse model of KRAS-driven LUAD, the loss of stress-induced metabolic regulators, regulated in development and DNA damage responses 1, can induce a HIF-dependent lipid storage pathway, which induces the FA transporter CD36 and thereby uptake of exogenous fatty acid[75]. KRAS mutation has been shown to promote FA synthesis through inducing FASN and stearoyl-CoA desaturase mediated by ERK2[76]. Besides, the FA metabolism has been reported to be upregulated by the PI3K-mTOR pathway[77], which has already been demonstrated to be regulated by KRAS[78]. The active AKT can facilitate both the ACLY and mTORc1. ACLY promotes the transformation of citrate to acetyl-CoA, which subsequently boosts lipogenesis. Active mTORc1 can activate slicing factor serine/threonine-protein kinase (SRPK2) and transcriptional factor sterol regulatory element binding protein-1c in the nucleus, which ensures the expression of a series of lipogenesis enzymes, including ACLY, FASN, *etc*.[79-81]. Besides promoting FAS, KRAS mutation has also been reported to suppress the oxidation of lipids. The suppression of hormone-sensitive lipase (HSL) by KRAS mutation leads to the decreased β-oxidation of FAs, which results in the accumulation of FAs[82]. STK11 deficiency has been found to cause the downregulation of FAO upregulation of fatty acid synthesis via activation of acetyl-CoA carboxylase due to the low level of phosphorylation of AMPK[83]. Kelch like ECH associated protein 1 (KEAP1) is another frequently observed co-mutation along with $KRAS^{mut}/STK11^{-/-}$. KEAP1 mutation has been reported to decrease lipid peroxides via enhancing the transcription factor nuclear factor, erythroid 2 like 2, which directly upregulates the suppressor of ROS, glutathione peroxidase 4[74,84,85]. The accumulated FAs in the TME have been found to cause the dysfunction of immune cells in KRAS-mutant mice model[86]. In the infiltrated cytotoxic $CD8^+$ T cells, the long-chain fatty acids are unbreakable and can result in a reduction of very-long-chain acyl-CoA dehydrogenase and lipid toxicity in T cells, which cause the exhaustion of infiltrated T cells[87]. In addition, the excess FAs caused by immunoglobulin-like transcript 4/paired immunoglobulin-like receptor B via mitogen-activated protein kinase 1/ERK1/2 signaling has been demonstrated to cause the senescence of effector T cells[88]. Moreover, the absorption of the FA-carrying tumor-derived exosomes by DCs can generate increasing storage of FAs and the enhancement of β-oxidation. The shift of mitochondrial oxidative phosphorylation due to the fuel alteration results in the dysfunction of DCs[89].

Previous studies have revealed that the accumulated FAs provides sufficient raw material for PGE2 production[90,91], and KRAS mutation can also enhance the production of PGE2 via upregulation of COX-2[92]. In NSCLC, PGE2 has been found to induce forkhead box P3 (FOXP3) expression and subsequently promotes the activity of $CD4^+$ and $CD25^+$ Tregs[93]. In metastatic murine renal carcinoma, overproduced PGE2 has also been reported to suppress the anti-tumor cytotoxic T cell lymphocyte responses by preventing the production of IFN-γ[94]. Moreover, the excess PGE2 in TME suppresses the production of X-C motif chemokine ligand 1 and CCL5 by NK cells and further blocks the recruitment of the conventional type 1 dendritic cells (cDC1s) through downregulating the respective receptors on cDC1s[95]. Increasing evidence indicates that the accumulated PGE2 in tumor cells exerts an immunosuppressive TME.

Cholesterol is normally synthesized from acetyl-CoA. Recent studies have found that cholesterol exerts an immunosuppressive TME by promoting the expression of immune checkpoint on $CD8^+$ T cells, which induces ER stress and consequently exhaustion of those T cells[96,97]. In KRAS-driven NSCLC, the synthesis of cholesterol has been found to be induced by Myc proto-oncogene protein (MYC) activation. The activation of MYC leads to an accumulation of cholesteryl esters (CE) stored in LDs and the increased

influx and the decreased efflux of cholesterol in tumor cells. Of note, the deactivation of MYC following activation gives rise to an additional increase of CEs[98].

In conclusion, there is supporting evidence indicating that FA metabolism can be induced by KRAS mutation through regulating various molecules. The excessive synthesis of FA and associated metabolites impedes the normal function of immune cells.

Glutamine and tryptophan metabolism

In normal cells, amino acid metabolism facilitates cells with energy and material for the biosynthesis of macromolecules, such as proteins and nucleotides. Amino acid metabolism reprogramming is required for hyperactive tumor cells to meet the high demand of energy and proteins required for cell proliferation[99]. Among the 21 amino acids within the human body, glutamine and tryptophan have been broadly reported to be rewired in various tumor cells and build an immune suppressive TME, which facilitates tumor cells to evade immune surveillance.

Glutamine can be successively transformed to glutamate, α-ketoglutarate, oxaloacetate, and aspartate by the catalyzation of glutaminase (GLS1), glutamate dehydrogenase 1, and glutamic-oxaloacetic transaminase 1/2, respectively[100]. KRAS mutation in NSCLC enhances the glutamine metabolism by inducing glutamine uptake, which results in a glutamine deficiency in TME[101]. KRAS mutation has been found to induce multiple glutamine transporters, such as solute carrier family 1 member 5 (SLC1A5) in NSCLC[102,103], solute carrier family 7 member 5 (SLC7A5)[104], and solute carrier family 38 member 2 (SLC38A2) via activation of hippo effector yes1 associated transcriptional regulator (YAP1)[103]. The upregulated transporter SLC1 increases the influx of glutamine, and the increased antiporter SLC7A effluxes glutamines and intakes other essential amino acids. The essential amino acids transported by SLC7A induce mTORC1-ribosomal protein S6 kinase B1 signaling and further promote protein synthesis[103,104]. It has also been shown that the mRNA level of GLS is higher in KRAS-mutated NSCLC, which indicates a higher consumption of glutamine in NSCLC[102]. Additionally, the $KRAS^{mut}/STK11^{-/-}/KEAP1^{-/-}$ co-mutant NSCLC was found dependent on glutaminolysis for fuel and was specifically sensitive to GLS inhibitors[85]. The high uptake of glutamine in tumor cells leads to a glutamine deficiency in TME, which causes immune suppression through blocking active T cells or producing immune suppressive cells. One study illustrated that the lack of glutamine results in the increase of HIF and then the rising secretion of IL-23 by macrophages, which promotes the proliferation of Tregs through the STAT3 signaling pathway. Tregs subsequently secretes TGF-β and IL-10 and suppresses the cytotoxicity of T cells[105]. Besides the shortage of glutamine in the TME, other conditions can also result in glutamine deprivation in tumor infiltrated immune cells. One study showed that the shortage of glucose causes defective N-linked glycosylation and endoplasmic reticulum stress and accordingly activates the endoplasmic reticulum to nucleus signaling 1 (IRE1) - X-box binding protein 1 (XBP1) axis. Activated XBP1 represses the glutamine transporters through post-translational regulation, which blocks glutamine uptake. The low level of glucose and glutamine causes the dysfunction of mitochondria, which impedes the production of IFN-γ by active T cells[106]. Moreover, glutamine deprivation increases the secretion of G-CSF and GM-CSF through activating the IRE1α/c-Jun N-terminal kinase pathway in cancer cells, which manipulates the formation of immunosuppressive MDSCs in TME[107]. However, the results from one study also indicate that high glutamine in TME tends to polarize the macrophages to pro-tumorigenic M2 type and inhibit the differentiation of the M1 macrophage[108], which is contrary to the conclusion from the above research. The contradictory conclusion of deprived glutamine indicates the complexity of glutamine in regulating TME.

Tryptophan can either be catalyzed to 5-hydrooxytryptophan, serotonin, and 5-hydroxyindoleacetic acid by tryptophan hydroxylase 1 and 2 (TPH1, TPH2), dopa decarboxylase, and monoamine oxidase A and B, respectively, or be catalyzed to kynurenine, kynurenic acid by indoleamine 2,3-dioxygenase 1 and 2 (IDO1/2), and tryptophan 2,3-dioxygenase (TDO2). In NSCLC, the KRAS mutation has been shown to reprogram tryptophan metabolism through upregulating the expression of immune checkpoint markers IDO1 according to microarray data[109]. The detailed mechanism remains to be discovered. The tryptophan metabolism has a pivotal role in promoting immune evasion. In tumor cells, tryptophan is transformed into kynurenine by IDO1. The upregulated IDO1 level induces excess kynurenine, which could be transferred to TME to promote Tregs and suppress effector T cells[110]. It has been illustrated that the activity of IDO can reflect the advanced disease, tumor metastasis, and immunotherapy responses to PD-1 inhibitors. The high expression of IDO can recruit immunosuppressive MDSCs through Tregs, which is Foxp3 dependent[111,112].

In summary, cancer cells can rely on glutamine as the main source of fuel and the precursor of other biomacromolecules. The deficiency of glutamine and increase of metabolite from tryptophan metabolism in TME give rise to an immunosuppressive phenotype. KRAS has been found to modulate several signaling pathways involved in glutamine metabolism, whereas further work is required to illustrate the more detailed mechanisms.

Besides the metabolism mentioned above, mutant KRAS has also been reported to regulate additional metabolic signaling pathways, including asparagine metabolism[113], authophagy[46,114], mcropinocytosis[115], *etc*. More detailed reviews of the metabolic rewiring driven by KRAS mutation can be found elsewhere[46,116-118]. Nutrient scavenging is a practical method for both tumor cells and immune cells to grab necessities from TME. Tumor cells and immune cells often outcompete stromal cells for nutrients or metabolites in TME, which leads to a nutrient-deprived environment[43]. However, most nutrients and some metabolites are also essential for the biosynthesis of macromolecules and production of energy craved by immunocytes[56,57,61,119,120]. Studies have also asserted that excess metabolites, such as lactate, alter the environment and affect the functions of immune cells[60,86,87,89,121]. A more comprehensive description of cancer metabolic reprogramming and immune response can be found in other reviews[122-125]. Collectively, the limited nutrients and redundant metabolites arouse the dysfunction in immune cells, which is one of the leading causes of resistance to ICIs. Taken together, KRAS mutation rewires the metabolic network in TME and thereby might cause a poor response to ICIs. A schematic figure of KRAS-driven metabolic signaling pathways in cancer cells is shown in Figure 2.

PROSPECTIVE STRATEGIES FOR OVERCOMING RESISTANCE TO ICIS

According to the ASCO guidelines, five main therapeutic strategies are applied in NSCLC: surgery, radiation therapy, chemotherapy, target therapy, and immunotherapy. The therapeutic schedule depends mostly on the stage and mutation type. As illustrated above, the efficacy of monotreatment of immunotherapy is controversial in NSCLC patients; thus, researchers have begun investigating the combinational strategy of immunotherapy and other therapies. In this section, we discuss the promising combined treatments of immunotherapy and other therapies in preclinical and clinical studies.

It is discussed above that PD-1/-L1 expression plays an essential role in response to ICIs. Therefore, targeting pathways modulating PD-1/-L1 expression might show potential for overcoming the resistance to ICIs, which has been demonstrated in several studies. In the KRAS-mutant lung cancer model, blockade of both PD-1 and helix-loop-helix transcription factor inhibitor of differentiation 1 knock out significantly enhances the amount of $CD8^+$ T cells as well as the expression of PD-L1, which impairs the tumor growth and increases the survival[126]. In other studies of $KRAS^{mut}$ lung cancer, anti-PD-1 combined with inhibition

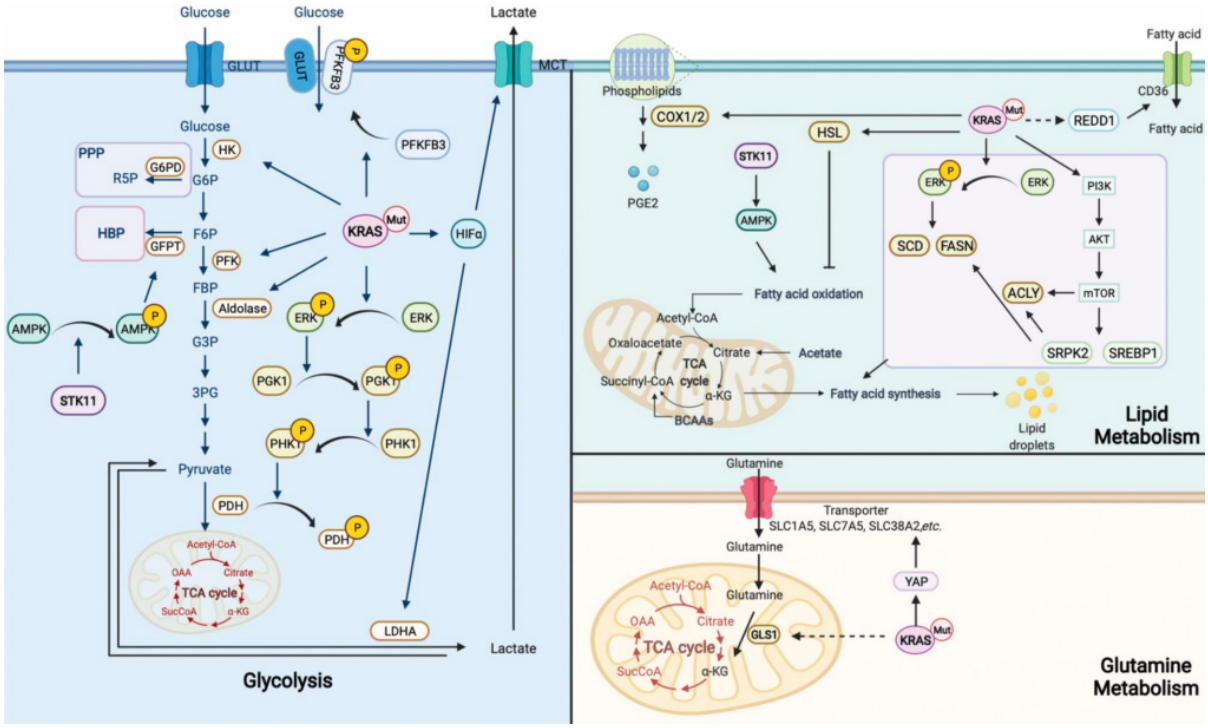

Figure 2. A schematic figure of KRAS-driven signaling pathways in the regulation of metabolic signaling pathways. GLUT: Glucose transporters; HK: hexokinase; PFK: phosphofructokinase; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; AKT: serine/threonine kinase; MAPK: mitogen-activated protein kinase 1; ERK: extracellular signal-regulated kinase 2; PDH: pyruvate dehydrogenase; PGK: phosphoglycerate kinase 1; HIF1α: hypoxia inducible factor 1 subunit alpha; JNK: mitogen-activated protein kinase 8; mTOR: mechanistic target of rapamycin kinase; LDHA/B: lactate dehydrogenase A/B; YAP: yes1 associated transcriptional regulator; IRF2: interferon regulatory factor 2; STAT3: signal transducer and activator of transcription 3; NF-κB: transcription factor P65; HSL: hormone-sensitive lipase; COX: cyclooxygenase; SCD: stearoyl-CoA desaturase; FASN: FA synthetase; ACLY: ATP-citrate lyase; GLS: glutaminase.

of the AKT-mTOR pathway by mTOR inhibitor or the STAT3 pathway by natural compound luteolin can remarkably decreased the expression of PD-1 or PD-L1, respectively, which consequently surmounts the resistance to anti-PD-1[127,128].

Other potential strategies to overcome the ICIs resistance include promoting the proliferation and activation of cytotoxic T cells and thwarting immunosuppressive immune cells, among others. The cholesterol-lowering drug statin has been reported to inhibit prenylation of KRAS and cause ER stress and immunogenic cell death, which cross-primes DCs and provokes $CD8^+$ T cells. Combinational treatment of anti-PD-1, statin, and oxaliplatin has shown the potential to overcome resistance to anti-PD-1[129]. In the $KRAS^{mut}/TP53^{-/-}$ lung cancer mouse model, chemokine CCL7 has also been found to recruit $CD11c^+$ or $Zbtb46^+$ DCs and promote T cell expansion, which increases the immune responses. When combined with anti-PD-1, CCL7 profoundly increases the survival of the NSCLC mouse model[130]. The BET bromodomain inhibitor has also been demonstrated to increase the number of infiltrated T-helper type 1 cells and reduce the population of Tregs, which augments the anti-tumor response to anti-PD-1[131]. Utilization of antibodies or natural compounds to block immunosuppressive cytokines or chemokines has been reported to hamper the resistance to ICIs in KRAS-mutant NSCLC mouse models[41,132].

Due to the approval of the $KRAS^{G12C}$ inhibitor AMG 510 in $KRAS^{G12C}$ NSCLC by the FDA, studies have been investigating the effect of the combination of KRAS inhibitors and ICIs at the bench and in clinics. In the

$KRAS^{G12C}$ CT26 syngeneic mouse model, AMG510 treatment has been found to induce a pro-inflammatory microenvironment by enhancing the tumor infiltrated $CD8^+$ T cells, DCs, NKs, *etc*., and it exerted extended anti-tumor efficacy when combined with anti-PD-1[133]. A phase 1 clinical study designed to evaluate the safety and efficacy of AMG 510 and immunotherapy is in process now (NCT03600883). In the $KRAS^{G12C}$ CT26 syngeneic mouse model, KRAS inhibitor MRTX849 combined with anti-PD-1 has also been shown to increase major histocompatibility complex I expression and decrease immunosuppressive factors, which increased PFS compared to monotherapy[134]. Phase 1/2 studies evaluating the safety, tolerability, PK, and clinical activity of MRTX849 in combination with pembrolizumab in patients with NSCLC are now recruiting (NCT04613596 and NCT03785249).

Other attempts have been made to combine ICIs with inhibitors targeting KRAS downstream. A phase 1/2 clinical trial has investigated the efficacy of the PLK inhibitor rigosertib combined with nivolumab in NSCLC patients with KRAS mutation (NCT04263090). The early results indicate that 29% (2/7) of patients achieved partial responses, most of whom harbor a $KRAS^{G12C}$ or $KRAS^{G12V}$ mutation; 43% (3/7) of patients had a stable disease; and 57% (4/7) of patients had progressive disease[135]. Studies on the combinational efficacy of other $KRAS^{G12C}$ inhibitors and ICIs, such as GDC-6036 (NCT04449874) and JDQ-443 (NCT04699188), are now recruiting. The efficacy of ICIs combined with tumor vaccine mRNA-5671, which specifically target $KRAS^{G12C/D/V}$ and $KRAS^{G13D}$, is now under investigation (NCT03948763).

Combination treatments which are not specifically for KRAS-mutant NSCLC have also received positive results in the NSCLC group. Multiple ICI treatment has shown promising results in NSCLC patients. In a phase 3 trial, patients were designed to receive mono-nivolumab, nivolumab combined with ipilimumab, nivolumab combined platinum-based chemotherapy, or mono-chemotherapy. The nivolumab combined with ipilimumab treatment group (medium OS = 17.1 months; 95%CI: 15.0-20.1) has shown longer median duration of overall survival compared to the chemotherapy treatment group (medium OS = 14.9 months; 95%CI: 12.7-16.7) among patients with PD-L1 (TPS ≥ 1%). The two-year overall survival rates were 40% and 32.8% and the median duration responses were 23.2 and 6.2 months, in the nivolumab combined with ipilimumab treatment group and chemotherapy treatment group, respectively. Patients with a PD-L1 (TPS < 1%) also benefit from nivolumab combined with ipilimumab (medium OS = 17.2; 95%CI: 12.8-22.0) compared with chemotherapy treatment (medium OS = 12.2 months; 95%CI: 9.2-14.3)[136]. In a phase 2 clinical trial, 97 patients were enrolled to study whether stereotactic body radiotherapy (SBRT) enhances the tumor responses to pembrolizumab. The results show that the ORR at 12 weeks was 36% in experimental arm (SBRT plus pembrolizumab) *vs.* 18% in control arm (pembrolizumab), the median PFS was 6.6 months (SBRT plus pembrolizumab, 95%CI: 4.0-14.6) *vs.* 1.9 months (pembrolizumab, 95%CI: 1.7-6.9), and the median OS was 15.9 months (SBRT plus pembrolizumab, 95%CI: 7.1 to not reached) *vs.* 7.6 months (pembrolizumab, 95%CI: 6.0-13.9)[137]. A phase 2 trial enrolled 96 patients to study the efficacy of combinational anti-PD-1 (SHR-1210) and anti-VEGFR (Apatinib) in NSCLC patients with wild-type EGFR and ALK (NCT03083041). The ORR was 29.7% and DCR was 81.3%. Further analysis showed that the ORR reached 50% in the patients with high TMB, indicating that this combo-treatment is acceptable in NSCLC patients with high TMB[138].

All the clinical trials mentioned above have illustrated good outcomes comparing with mono-immunotherapy in NSCLC, regardless of KRAS status. Further umbrella analyses are still needed to elucidate the combo-treatment efficacy on the KRAS-mutant NSCLC groups. All clinical trials are listed in Table 1.

Table 1. Immune checkpoint inhibitors active in clinical trials

Immune checkpoint inhibitors	NCT identifier numbers	Combined therapy	Target	Allocation	Phase	Size	Primary ends	Status
Anti-PD-1/-L1	NCT03600883	AMG 510	$KRAS^{G12C}$	Randomized	Phase 1	733	AEs, DLTs, significant clinical changes	Recruiting
Atezolizumab	NCT04449874	GDC-6036 bevacizumab, cetuximab, rrlotinib	$KRAS^{G12C}$ VGFR EGFR TKI	Non-randomized	Phase 1	236	AEs, DLTs	Recruiting
Pembrolizumab	NCT04429542	BCA101	EGFR/TGF-β	Non-randomized	Phase 1	292	Safety, Cmax, PFS, AEs, ORR, OS, DLTs, MTD	Recruiting
	NCT04613596	MRTX849	$KRAS^{G12D}$	N/A	Phase 2	120	Clinical activity	Recruiting
	NCT03785249	MRTX849	$KRAS^{G12D}$	N/A	Phase 1	565	Safety, tolerability, drug levels, molecular effects, and clinical activity	Recruiting
	NCT03299088	Trametinib	MEK	Non-randomized	Phase 1	15	DLTs, ORR, PFS	Active, not recruiting
	NCT02779751	Abemaciclib Anastrozole	CDK Estrogen synthesis	Non-randomized	Phase 1	100	PFS, AEs, ORR, OS, DCR, PK	Active, not recruiting
	NCT03948763	mRNA-5671/V941	Vaccine	Non-randomized	Phase 1	100	DLTs, AEs, ORR	Recruiting
	NCT04340882	Docetaxel Ramucirumab	Chemotherapy VEGFR	N/A	Phase 2	41	PFS, AEs, ORR, OS	Recruiting
	NCT03225664	Trametinib	MEK	N/A	Phase 1/2	37	ORR	Active, not recruiting
	NCT02492568	Radiation		N/A	Phase 2	96	ORR, toxicity	Complete
Nivolumab	NCT04263090	Rigosertib	PLK1	N/A	Phase 1/2	30	MTD, ORR, PFS, OS	Recruiting
	NCT02852083	Pioglitazone Clarithromycin	PPARγ Bacteria proteins	Randomized	Phase 2	86	PFS, AEs, ORR, OS	Unknown status
	NCT02492568	Ipilimumab	Anti-CTLA-4	N/A	Phase 3	1980	OS, ORR, disease related symptom	Recruiting
SHR-1210	NCT03083041	Apatinib	Anti-VEGFR	N/A	Phase 2	117	AEs, ORR	N/A

AEs: Adverse events; DLTs: dose-limiting toxicities; PFS: progression-free survival; OR: overall survival; RR: response rate; MTD: maximum tolerant doses; ORR: objective response rate; DCR: disease control rate; PK: pharmacokinetics; Cmax: plasma concentration.

The application of dietary modifications to supplement other cancer therapies is drawing more attention currently. The intake of different nutrients might alter the nutrient availability in the plasma, thus the TME[139]. Preclinical and retrospective studies have assumed that cancer development and prognosis are negatively correlated to unhealthy diets, such as diets high in sodium and fat[121,140]. Growing research has found that restriction or supplementation of specific metabolites might ameliorate therapeutic responses. The manipulation of nutrient accessibility remarkably reprograms the metabolic activity and therefore leads to alterations in cell activities and sensitivity to therapies, which is mainly modulated by nutrient-sensing pathways[141-144]. The dietary modifications enhance cancer therapy through sophisticated mechanisms, which are well summarized in other reviews[145-147]. As mentioned above, glucose is the primary source for not only energy but also metabolic intermediates for macromolecule synthesis for both tumor cells and immune cells. A diet low in glucose but normal in calories can reduce the blood glucose and decelerate tumor growth in some tumor models[148]. However, the glucose restriction might also affect the normal

function of immune effect cells. During the process of proliferation, differentiation, and activation of immune cells, the requirement for glucose might differ. Studies have found that inhibition of glycolysis restrains the differentiation of $CD4^+$ T cells to Treg cells and promotes the differentiation of activated $CD8^+$ T cells to long-lived memory $CD8^+$ T cells[119,149]. Accordingly, we presume that timing and personalized diet should be taken into account when applying glucose restriction to supplement ICI treatment. Likewise, the lipid and amino acid metabolisms function differently at different stages of differentiation and activation of immune cells, thus the need for nutrients varies[120,150-152]. Therefore, it is critical to understand the metabolic network and landscape in TME in KRAS-driven NSCLC and how they affect the behavior of tumor cells and the function of stromal cells. Moreover, the impact of dietary modification on ICI response might be influenced by patient-specific variables. Many studies are currently investigating the molecular mechanisms and evaluating the effects of dietary interventions in enhancing cancer therapies. Collectively, dietary modification is a promising strategy for overcoming resistance to or improving the efficacy of ICI treatment in KRAS-mutant NSCLC.

CONCLUSION

Further understanding of the resistance mechanisms to ICIs mediated by KRAS mutation in NSCLC could provide implications on prospective therapeutic interventions to overcome the resistance or improve the efficacy. This will need further investigations to unearth metabolic pathways regulated by specific KRAS mutations, as well the modulatory effect on shaping TME directly and indirectly. Additional attempts to identify metabolic signaling pathways that promote immunosuppressive TME and resistance to ICIs will help discover the targetable metabolic vulnerabilities to improve the efficacy or overcome the resistance to ICIs through dietary modifications.

DECLARATIONS

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Figure 2 was illustrated using BioRender.com.

Authors' contributions

Research, image illustration and contributed to writing of review manuscript: Li Y, Hu L
Performed data acquisition for tables: Li Y, Peng X, Xu H, Tang B
Substantial contributions to conception and planning of review and editing: Hu L, Xu C
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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Mechanism underlying the immune checkpoint inhibitor-induced hyper-progressive state of cancer

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Abstract

Immune checkpoint inhibitors (ICIs) are gradually replacing chemotherapy as the cornerstone of the treatment of advanced malignant tumors because of their long-lasting and significant effect in different tumor types and greatly prolonging the survival time of patients. However, not all patients can respond to ICIs, and even rapid tumor growth after treatment with ICI has been observed in a number of clinical studies. This rapid progression phenomenon is called hyper-progressive disease (HPD). The occurrence of HPD is not uncommon. Past statistics show that the incidence of HPD is 4%-29% in different tumor types, and the progression-free survival and overall survival of patients with HPD are significantly shorter than those of the non-HPD progressor group. With the deepening of the study of HPD, we have established a preliminary understanding of HPD, but the diagnostic criteria of HPD are still not unified, and the addition of biomarkers may break this dilemma. In addition, quite a few immune cells have been found to be involved in the occurrence and development of HPD in the tumor microenvironment, indicating that the molecular mechanism of HPD may be triggered by a variety of ongoing events at the same time. In this review, we summarize past findings, including case reports, clinical trials, and fundamental research; compare the diagnostic criteria, incidence, and clinical prognostic indicators of HPD in different studies; and explore the molecular mechanism and future research direction of HPD.

Keywords: Immune checkpoint inhibitors, hyper-progressive disease, immunotherapy, tumor microenvironment

INTRODUCTION

Immune checkpoint inhibitors (ICIs) have continuously promoted the progress of the treatment of malignant tumors since their advent. They have gradually replaced chemotherapy as the cornerstone for the treatment of malignant tumors; however, ICIs are only effective in some patients and remain ineffective in most populations. Changes in the tumor microenvironment (TME) induced by ICIs stimulate the accelerated growth of malignant tumor cells. This special tumor progression mode is called the hyper-progressive disease (HPD) state. Lahmar *et al.*[1] reported the HPD phenomenon for the first time in a wall newspaper at the 2016 European Society of Medical Oncology Annual Meeting. Eight patients with advanced non-small cell carcinoma (NSCLC) exhibiting fast progression at the time of initial examination were identified as HPD cases. HPD gained attention in 2017 when Champiat *et al.*[2] reported a 9% HPD incidence in 131 cancer patients in a phase I prospective study. Evidence of HPD, the phenomenon of early crossover of the survival curve, is also reported in some phase III clinical studies, including in NSCLC (CheckMate026[3], CheckMate057[4], and CheckMate227[5]), HNSCC (CheckMate141[6]), and uroepithelial carcinoma (Keynote045[7] and IMvigor211[8]). Patients receiving immunotherapy died at a greater rate in the first three months than those treated with chemotherapy. HPD is not unique to immunotherapy and can also be caused by chemotherapy[9] and targeted therapy[10]. However, the incidence of HPD after ICI treatment is significantly higher than in the chemotherapeutic regime[11]. Since its discovery in 2016, several studies on HPD have been reported in the last five years. Nevertheless, the incidence, diagnostic criteria, and pathogenesis of HPD remain in the preliminary stages. This review summarizes the recently published cases, clinical studies, and basic studies on HPD.

DIAGNOSTIC CRITERIA FOR HPD

At present, there is no agreement on the diagnostic criteria of HPD. Although many clinical studies on HPD adopt different diagnostic criteria, the diagnostic indicators of HPD mainly focus on the following five: tumor growth rate (TGR), ΔTGR, tumor growth kinetics (TGK), Response Evaluation Criteria in Solid Tumors (RECIST), and time to failure (TTF). TGR represents the percentage of monthly tumor volume growth (excluding new and immeasurable lesions), and the difference between the two at and before treatment is defined as ΔTGR. TGK is defined similarly to TGR, but it primarily reflects tumor growth rate per unit time. TTF refers to the time of treatment failure. Champiat *et al.*[2] earlier adopted such criteria as TGR > 2 and RECIST to assess the progress for the first time to define HPD. In the same year, Ferrara *et al.*[9] used a different cut-off value of ΔTGR > 50%. Kato *et al.*[12] added TTF < 2 months on the basis of predecessors. Saâda-Bouzid *et al.*[13] used a new index, TGK, as a measure of tumor growth rate that may be more appropriate to define HPD. Kim *et al.*[14] reviewed the survival time of 335 patients with advanced NSCLC who received ICI monotherapy; it was proved that HPD defined by volume measurement (TTF < 2 months, TGK > 2, and ΔTGR > 50%) is more accurate than that defined based on one-dimensional analysis (RECIST 1.1). Kas *et al.*[15] conducted a retrospective study of 406 patients with advanced NSCLC treated with ICIs. They calculated their results using the different definitions of five clinical studies. The incidence of HPD ranged from 5.4% to 18.5%, and the median survival ranged from 3.4 to 6.0 months. Δ TGR was found to be most correlated with poor prognosis, and ΔTGR > 100% was updated as the optimal threshold.

Although the volumetric method is superior to the RECIST standard, there are practical problems: first, not all patients can complete the pre-baseline computed tomography (CT) scan, especially those receiving ICI as late first-line treatment. Second, new and unmeasurable lesions cannot be measured by TGR.

Matos *et al.*[16,17] returned to RECIST standard and proposed a new method to define HPD: (1) target lesions increased by more than 40% from baseline; and/or (2) target lesions increased by more than 20% from baseline and new lesions appeared in at least two different organs. The overall survival (OS) of the HPD group using the new standard decreased significantly, which was statistically significant, compared to the non-HPD group, whereas the OS of the HPD group using TGR decreased, but not statistically significantly. However, Gomes da Morais *et al.*[18] reviewed the literature and compared the main criteria of HPD proposed by Ferté, Le Tourneau, Garralda, and Caramella. These criteria include ΔTGR > 100 (Caramella) and 20% target lesion progression plus the occurrence of new lesions in at least two different organs. The incidence of HPD was 23.9%, 23.9%, 32.4% and 8.4%, respectively. They believed that the Caramella standard has low sensitivity; the Garralda standard has low specificity; and the Le Tourneau and Ferté standards seem to have similar performance in detecting HPD, but, from a practical point of view, the two-dimensional evaluation of TGK (Le Tourneau) is easier than the three-dimensional evaluation of TGR (Ferté). The importance of pre-baseline CT scanning in diagnosing HPD was thus highlighted, but only 71 eligible patients were enrolled in this study. Later, Abbar *et al.*[19] expanded the study to 169 advanced NSCLC patients treated with ICI; the incidence of HPD (11.3%, 5.7%, 17.0%, 9.6% and 31.7%) was calculated based on five indicators. In addition to the discovery of large heterogeneity, the definition of HPD based on TTF standard was correlated with OS, while the other diagnostic criteria were not correlated with OS.

Thus, combining indicators with each other may be more conducive to diagnosis. The radiological and clinical diagnostic criteria for HPD are still being explored. With the deepening of the understanding of biomarkers for HPD, biomarkers may be involved in the diagnostic criteria of HPD in the future, and the joint definition of HPD by three diagnostic methods may be more accurate and practical.

INCIDENCE AND PROGNOSTIC INDICATORS OF HPD

The incidence and clinical prognostic indicators of HPD are also different. Chen *et al.*[20] reviewed the medical records of 377 patients with multiple malignancies and reported the incidence of HPD (10.08%). Factors associated with HPD include the presence of more than two metastatic sites, Eastern Cooperative Oncology Group score ≥ 2, liver metastasis, and lactic dehydrogenase level higher than the normal upper limit. Kirsten rat sarcoma viral oncogene homolog status is significantly correlated with HPD in colon cancer patients. Two large-scale meta-analyses reported the incidence of HPD in patients with pan-cancer as 1%-30%[21] and 5.9%-43.1%[22]. The clinical prognostic markers used in these analyses were similar to those reported by Chen *et al.*[20]. Ferrara *et al.*[9], using RECIST 1.1 and TGR criteria, reported a 13.8% (56/406) HPD incidence in patients with advanced NSCLC; HPD was associated with more than two metastases before immunotherapy. Kim *et al.*[23] first defined three criteria (TGR, TGK, and TTF) to calculate the incidence of HPD (20.9%, 20.5%, and 37.3%, respectively). In HPD patients who satisfied both TGR and TGK criteria, poorer progression-free survival (PFS) and OS were observed. Although no clinicopathological variables of HPD were reported in the study, in the exploratory biomarker analysis of peripheral blood, $CD8^+$ T lymphocytes, lower effector/memory subsets ($CCR7^-CD45RA^-$ T cells in total $CD8^+$ T cells), and higher populations of severely depleted cells ($TIGIT^+$ T cells in $PD\text{-}1^+CD8^+$ T cells) were associated with HPD and poor survival. In two real-world studies, the incidence of HPD in advanced NSCLC was 19.2% (16/83)[24] and 8.1% (6/74)[25]. Among them, one study reported an increased rate of fluid accumulation (up to 90%) and decreased albumin level, while the other showed a significant increase in the number of circulating Treg cells in HPD patients. Chen *et al.*[26] performed a meta-analysis consisting of 1389 NSCLC patients from six clinical studies and found that the incidence of HPD was 8.02%-30.43%. The incidence of HPD and clinical prognostic indicators in cancer types are shown in Table 1.

Table 1. Recent retrospective studies on hyper-progression after immunotherapy

Tumor type	Agents	HPD criteria	HPD incidence	Prognostic indicators	Outcomes (HPD *vs.* non-HPD)	Ref.
Multiple tumor types	PD-1/PD-L1 inhibitor monotherapy	-	1%-30% (217/1519)	Serum LDH > upper normal limit; > 2 metastatic sites prior to immunotherapy; liver metastatic sites; RMH prognostic score ≥ 2; positive PD-L1 expression status	-	Kim *et al.*[21] (2019)
Multiple tumor types	PD-1/PD-L1 inhibitor monotherapy	RECIST criteria (1.4× baseline sum target lesions or 1.2× baseline sum target lesions + new lesions in at least 2 different organs) or TGR ≥ 2	RECIST criteria, 10.7% (29/270); TGR criteria, 6.3% (14/221)	RECIST criteria of no or TGR criteria of liver metastatic sites; > 2 metastatic sites prior to immunotherapy	OS: 5.23 months *vs.* 7.33 months, $P = 0.04$, by RECIST; 4.2 months *vs.* 6.27 months, $P = 0.346$, by TGR	Matos *et al.*[17] (2020)
Multiple tumor types	PD-1 inhibitors (nivolumab or pembrolizumab)	ΔTGR > 50%	10.08% (38/377)	> 2 metastatic sites prior to immunotherapy; ECOG ≥ 2; hepatic metastases; serum LDH > upper normal limit; KRAS status in colorectal cancer	OS: 3.6 months *vs.* 7.3 months, $P < 0.01$	Chen *et al.*[20] (2021)
Multiple tumor types	PD-1 or PD-L1 inhibitor monotherapy or combined with CTLA-4 inhibitor	4 categories (TGR, TGK, early tumor burden increase, or combinations of the above)	5.9%-43.1% (3109)	-	-	Park *et al.*[22] (2021)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy or combined with CTLA-4 inhibitor	RECIST 1.1 progression and ΔTGR > 50%	14% (56/406 treated with ICI); 5% (3/59 treated with chemotherapy)	> 2 metastatic sites prior to immunotherapy	OS: HR = 2.18, 95%CI: 1.29-3.69, $P = 0.03$	Ferrara *et al.*[9] (2018)
NSCLC	PD-1 inhibitors (nivolumab)	< 3 nivolumab injections	20% (57/292)	PS > 2 at nivolumab initiation	OS: 1.4 months *vs.* 13.5 months, $P < 0.0001$	Costantini *et al.*[112] (2019)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy	Volumetric time-dependent criteria (TGK ≥ 2) or one-dimensional criteria: RECIST 1.1 progression	14.3% (48/335 by volumetric assessment); 13.1% (44/335 by one-dimensional criteria)	High neutrophil-to-lymphocyte ratio; LKB1 mutation	OS: 4.7 months *vs.* 7.9 months, $P = 0.009$, by volumetric; 5.2 months *vs.* 7.1 months, $P = 0.288$, by RECIST	Kim *et al.*[14] (2020)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy	TGK ≥ 2, TGR ≥ 2, or TTF < 2 months	20.9% (55/263 TGK), 20.5% (54/263 TGR), 37.3% (98/263 TTF)	≥ 2 metastatic locations; liver metastases; neutrophils; neutrophil-to-lymphocyte ratio; LDH; high $CD8^+PD\text{-}1^+TIGIT^+$ T cells; low $CD8^+CCR7^-CD45RA^-$ T cells	PFS: HR = 4.62, 95%CI: 2.87-7.44, $P < 0.05$; OS: HR = 5.71, 95%CI: 3.14-8.23, $P < 0.05$	Kim *et al.*[23] (2019)
NSCLC	PD-1 inhibitors (nivolumab)	RECIST 1.1 progression and TGR ≥ 2	19.2% (16/83)	Pleura or pericardium metastasis; low circulating albumin	PFS: 0.43 months *vs.* 1.35 months; OS: 2.2 months *vs.* 4.1 months	Kas *et al.*[24] (2020)
NSCLC	PD-1 /PD-L1 inhibitor monotherapy or combined with other	Ferté criteria (RECIST 1.1 progression and TGR ≥ 2), Le Tourneau criteria (TGK > 2), Garralda criteria (increase of ≥ 20% in target tumor burden plus	5.4%-18.5% (406)	No (including previously described prognostic factors such as age, LDH, albumin, > 2 metastatic sites,	-	Kas *et al.*[15] (2020)

	immunotherapy treatments	multiple new lesions or increase of ≥ 40% in target tumor burden compared with baseline) or Caramella criteria (RECIST 1.1 progression and ΔTGR > 100%)		RMH score)		
NSCLC	PD-1 /PD-L1 inhibitor monotherapy or combined with other immunotherapy treatments	-	8.02%-30.43% (1389)	ECOG > 1; RMH ≥ 2; serum LDH > upper Normal limit; > 2 metastatic sites prior to immunotherapy; liver metastases	-	Chen *et al.*[26] (2020)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy or combined with CTLA-4 inhibitor	5 definitions (TGR, ΔTGR, TGK, RECIST, or TTF)	11.3%, 5.7%, 17%, 9.6%, 31.7% (169)	-	-	Abbar *et al.*[19] (2021)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy	TGK > 2 and TTF ≤ 2 months	11.3% (26/231)	Heavy smoker; PD-L1 expression ≤ 1%; ≥ 3 metastatic sites	OS: 5.5 months *vs.* 6.1 months	Kim *et al.*[110] (2021)
NSCLC	PD-1/PD-L1 inhibitor monotherapy or combined with chemotherapy	TGR > 2	17.6% (25/142 monotherapy); 2.9% (1/34 combination therapy)	-	-	Matsuo *et al.*[113] (2021)
NSCLC	PD-1 or PD-L1 inhibitor monotherapy	TGK ≥ 2	8.1% (6/74)	$CD4^+CD25^+CD127^{lo}FoxP3^+$ Treg cells was increased on Day 7 after initiation of treatment	-	Kang *et al.*[25] (2021)
HNSCC	PD-1 or PD-L1 inhibitor monotherapy or combined with CTLA-4 inhibitor	TGK > 2	14.4% (18/125)	Younger age; primary tumor of oral cavity; previous locoregional irradiation	PFS: 1.2 months *vs.* 3.4 months, $P < 0.001$; OS: 3.4 months *vs.* 10.7 months, $P = 0.047$	Park *et al.*[31] (2020)
HNSCC	PD-1 or PD-L1 inhibitor monotherapy or combined with CTLA-4 inhibitor	TGK ≥ 2	15.4% (18/117)	Primary site in the oral cavity; administration of ICI in the second/third setting	PFS: 1.8 months *vs.* 6.1 months, $P = 0.0001$; OS: 6.53 months *vs.* 15 months, $P = 0.0018$	Economopoulou *et al.*[51] (2021)
MM	PD-1 inhibitor, CTLA-4 inhibitor monotherapy or combination	TTF < 2 months, doubling of tumor burden, and TGR > 2	1.3% (1/75)	-	-	Schuiveling *et al.*[114] (2021)
GC	PD-1 inhibitors (nivolumab)	TGK ≥ 2 and $(S_{POST}/S_0\text{-}1) > 0.5$	22.1% (143)	PD-L1 CPS; MMR	PFS: 1.2 months *vs.* 1.7 months, $P < 0.001$; OS: 3.3 months *vs.* 6.8 months, $P = 0.012$	Hagi *et al.*[115] (2020)
HCC	PD-1 inhibitors (nivolumab)	TGK > 4 and ΔTGR > 40%	12.7% (24/189)	Neutrophil-to-lymphocyte ratio	PFS: HR = 2.194, 95%CI: 1.214-3.964; OS: HR = 2.238, 95%CI: 1.233-4.062	Kim *et al.*[116] (2021)
RCC and UC	PD-1/PD-L1 inhibitor monotherapy	Tumor burden increase ≥ 50%, TGR ≥ 2, or ≥ 10 metastatic sites	0.9% (1/102), 11.9% (12/101)	UC; creatinine > 1.2 mg/dL	PFS: 1.3 months *vs.* 3.9 months, $P < 0.001$; OS: 3.5 months *vs.* 7.3 months, $P < 0.001$	Hwang *et al.*[117] (2020)

GYN	PD-1 inhibitor	Tumor burden increase of ≥ 40% or tumor burden increase of ≥ 20% plus multiple new lesions	23.3% (14/60)	Neutrophil-to-lymphocyte ratio; > 3 - metastatic sites		Rodriguez Freixinos *et al.*[118] (2018)

PFS: Progression-free survival; OS: overall survival; NSCLC: non-small-cell carcinoma; HNSCC: head and neck squamous cell carcinoma; MM: malignant melanoma; GC: gastric cancer; HCC: hepatocellular carcinoma; RCC: renal cell carcinoma; UC: urothelial carcinoma; GYN: gynecological malignancies; PD-1/PD-L1: programmed cell death-1/programmed cell death-ligand 1; LDH: lactic dehydrogenase; RMH: Royal Marsden Hospital score; RECIST: Response Evaluation Criteria in Solid Tumors; TGR: tumor growth rate; ΔTGR: the difference of TGR before and during immunotherapy; TGK: tumor growth kinetics; TTF: time to treatment failure; ECOG: Eastern Cooperative Oncology Group; KRAS: Kirsten rat sarcoma viral oncogene homolog; CPS: combined positive score of PD-L1 expression; MMR: mismatch repair.

CASE SUMMARY

The limitations of ICIs, as they may not be appropriate for some patients, caused "disease flare" in a 54-year-old man with stage IIB lung adenocarcinoma after 10th-line treatment with nivolumab[27]. This case opened up the HPD patient reports, and, according to incomplete statistical data, in 44 cases involving 53 patients, malignant tumor types were mainly distributed in the respiratory system, digestive system, and urinary system and were immune to single and double drugs to a significantly higher degree than due to the immune or anti-angiogenesis drugs with combination chemotherapy. Most patients with HPD after ICI treatment developed liver, lung, and brain metastases. Selected case studies are listed in Table 2. Among them, the youngest patient was a 13-year-old girl suffering from malignant melanoma, which progressed to HPD mode after two cycles of treatment with avelumab in palliative radiotherapy. The Food and Drug Administration has approved ICIs for the treatment of children with microsatellite unstable malignant tumors based on reports in adults[28]. However, the interaction between children's immune systems and anti-PD1 therapy remains unclear. The oldest patient was an 80-year-old patient with lung squamous carcinoma[29]. The symptoms of HPD were pneumonia, pleural effusion, and pericardial effusion. Many patients developed the same symptoms after ICI treatment for malignant tumors of the respiratory system and digestive system and malignant melanoma. A previous study in South Korea reported a higher frequency of increased fluid accumulation in HPD patients with pleural or pericardial metastases after treatment with nivolumab as compared to the progressive disease (PD) patients without HPD [90% (9/10) *vs.* 28.6% (4/14); $P = 0.005$]; the circulating albumin level was significantly reduced in HPD patients ($P = 0.030$)[24]. A considerable proportion of HPD occurred in patients after radiotherapy, which suggested that radiotherapy had a bidirectional regulatory effect on the anti-tumor immune response. If the immunosuppressive function of radiotherapy is dominant, a combination of ICIs may lead to HPD[30]. A clinical study of head and neck squamous cell carcinoma also suggested that previous local irradiation was an important predictor of HPD[31]. In addition to being associated with radiotherapy, AKT1 E17K mutation[32] and PI3K/AKT pathway[33] were also related to HPD. Interestingly, after immunohistochemical staining of the primary tumor and metastases samples with HPD, Barham *et al.*[34] showed that the tumor infiltrating lymphocyte (TIL) number was not necessarily correlated with ICI response, as levels of granzyme B and TIA-1 of infiltrated $CD8^+$ T cells were mostly negative, indicating that these were inflammatory T cells which cause tumor drug resistance and myocarditis. They cannot effectively dissolve the tumor, so additional functional markers are required to distinguish between inflammatory and cytolytic $CD8^+$ TIL. For treatment, the salvage therapy in HPD has not been limited to chemotherapy. A patient with lung adenocarcinoma developed HPD with rib metastasis shortly after ICI-based combination therapy, and the lesion was significantly reduced after implantation of I^{125} particles into the chest wall[35]. Another patient with lung adenocarcinoma showed MET amplification on re-biopsy after HPD and remission occurred with a c-MET inhibitor[36]. A patient with triple-negative breast cancer showed HPD after pembrolizumab treatment combined with chemotherapy and remission with atezolizumab administration combined with chemotherapy[37]. A patient with cardiac cancer was in remission after salvage

Table 2. Cases summary on hyper-progression after immunotherapy

Tumor type	Gender	Age (years)	Agents	Radiotherapy before ICIs	Clinical symptoms	Progressive organ	Ref.
SCLC	Male	35	Nivolumab	No	Pleural effusion	Chest wall	Chiba *et al.*[119] (2020)
LUSC	Male, Male	69, 80	Nivolumab	No	Pneumonia, pleural effusion, pericardial effusion	Lung	Kanazu *et al.*[29] (2018)
LUAD	Female	66	Pembrolizumab	Yes	Pleural effusion, pericardial effusion	Brain, lung	Fricke *et al.*[120] (2020)
LUAD	Male	68	Nivolumab	No	Jaundice, fever	Liver, pancreas	Martorana *et al.*[121] (2021)
LUAD	Female	63	Sintilimab	Yes	Abdominal distension, poor appetite	Liver, pancreas	Lin *et al.*[122] (2020)
LUAD	Male	65	Pembrolizumab and paclitaxel liposome (salvage treatment: c-Met inhibitor)	Yes	-	Brain, lung	Peng *et al.*[36] (2020)
LPC	Male	66	Atezolizumab	Yes	Pericardial effusion, pericarditis, pleural effusion	Lung, brain, liver, diaphragm	Oguri *et al.*[123] (2021)
ESCC	Male	40	Camrelizumab	No	-	Liver	Wang *et al.*[124] (2020)
GC	Male	36	Nivolumab (salvage treatment: capecitabine and pyrotinib)	No	-	Lung, liver	Huang *et al.*[125] (2019)
AEG	Female	56	Pembrolizumab (salvage treatment: paclitaxel and ramucirumab)	No	-	Lung, spine, ilium, retroperitoneal lymph node, *etc.*	Sama *et al.*[38] (2019)
HCC	Male	36	Atezolizumab and bevacizumab	No	Abdominal pain	Liver	Singh *et al.*[126] (2021)
HCC	Male/Male/Male	69/72/69	Tremelimumab/nivolumab/tremelimumab and durvalumab	No/TARE/TARE	-	Liver, portal vein thrombosis/lung, peritoneum/liver, lung	Wong *et al.*[127] (2019)
COAD	Female	48	Pembrolizumab	No	Fatigue	Liver, retroperitoneal lymph node	Chan *et al.*[128] (2020)
CMM	Female	25	Nivolumab	Yes	Ascites, pleural effusion, epilepsy	Peritoneum, pleura, brain	Yilmaz *et al.*[129] (2019)
AMM	Female	49	Ipilimumab and nivolumab (salvage treatment: chemotherapy)?	No	-	Lung, brain	Forschner *et al.*[130] (2017)
MMM	Female	79	Ipilimumab and nivolumab	Yes	Fulminant myocarditis, ascites, dizzy	Lung, peritoneum	Barham *et al.*[34] (2021)
MM	Female	13	Nivolumab	Yes	-	Multiple organs	Vaca *et al.*[28] (2019)
IBC	Male	78	Nivolumab	Yes	-	Sternum, liver	Koukourakis *et al.*[131] (2020)
KIRC	Female	42	Nivolumab	Yes	Arthritis of hand and knee	Lung	Liu *et al.*[30] (2021)

mUC	Male	57	Anti-PD-L1 and immune checkpoint modulator	No	-	Liver, brain	Grecea *et al.*[132] (2020)
CSEC	Female	46	Pembrolizumab	Yes	Biliary obstruction	Liver	Lin *et al.*[122] (2020)
SCCC	Female	49	Pembrolizumab	No	-	Lung	Xu *et al.*[32] (2019)
PM	Male	75	Nivolumab	No	Abdominal distension	Liver	Ikushima *et al.*[133] (2020)
TNBC	Female	67	Pembrolizumab and gemcitabine (salvage treatment: atezolizumab and nab-paclitaxel)	No	Fatigue, poor appetite, abdominal pain	Liver	Feng *et al.*[37] (2021)
MSC	Female	60	Nivolumab	No	Decreased eyesight	Orbit, brain	Xiang *et al.*[134] (2020)
LS	Male	63	Durvalumab and tremelimumab	Yes	-	Liver	Chan *et al.*[135] (2020)

SCLC: Small cell lung cancer; LUSC: lung squamous cell carcinoma; LUAD: lung adenocarcinoma; LPC: lung pleomorphic carcinoma; ESCC: esophageal squamous cell carcinoma; GC: gastric cancer; AEG: adenocarcinoma of esophagogastric junction; HCC: hepatocellular carcinoma; COAD: colon adenocarcinoma; CMM: cutaneous malignant melanoma; AMM: acral malignant melanoma; MMM: mucosal malignant melanoma; MM: malignant melanoma; IBC: invasive bladder cancer; KIRC: kidney renal clear cell carcinoma; mUC: metastatic urothelial cancer; CSEC: cervical squamous epithelium carcinoma; SCCC: small cell carcinoma of cervix; PM: peritoneal mesothelioma; TNBC: triple-negative breast cancer; MSC: maxillary sinus carcinoma; LS: liposarcoma.

therapy with paclitaxel and ramucirumab following HPD[38].

MOLECULAR MECHANISM UNDERLYING HPD

The mechanism of action underlying ICI is the removal of the "braking" function of immune checkpoints and reduction in the escape of tumor cells to enhance the anti-tumor immune response of effector T cells[39]. ICIs reverse the immunosuppressive state of T cells by disrupting the programmed cell death-1/programmed cell death-ligand 1 (PD-1/PD-L1) axis[40]. However, PD-1 receptors are present not only on the surface of T cells but also on the surface of many innate or acquired immune cells, including NK cells, monocytes, macrophages, Treg cells, and B cells[41]. Furthermore, immune cells have varying impacts on PD-1/PD-L1 axis disruption, boosting or inhibiting immune function. In addition, tumor treatment through ICI intervention may also induce changes in the oncogenic pathways of the tumor cells and result in their rapid proliferation and spread[42]. Therefore, HPD may not be triggered by a single factor, but by a series of events that occur simultaneously. Most of the current studies on the molecular mechanisms of HPD focus on the tumor and the tumor microenvironment. In the next sections, we discuss these in detail to facilitate the understanding of the molecular mechanisms underlying ICI-induced HPD. The molecular mechanisms underlying HPD are shown in Table 3.

Alteration in the tumor cell types following ICI

HPD is a type of primary resistance to immunotherapy, and the mechanism of its occurrence involves alteration in the tumor cell types and the tumor microenvironment. These changes range from enhanced proliferative capacity, invasiveness, and drug resistance of tumor cells to a reduced immunosuppressive capacity in the tumor microenvironment. The tumor cells themselves are altered due to the following reasons: (1) loss of expression of

Table 3. Mechanisms summary on hyper-progression after immunotherapy

Tumor cells	Tumor microenvironment	
1. Loss of expression of tumor-associated antigens[43] 2. Impairment of antigen processing and delivery[44] 3. Persistent upregulation of PD-L1 expression on the surface of tumor cells[45] 4. Apoptotic resistance in tumor cells[46,47] 5. Induced dormancy and senescence of tumor cells[48] 6. Tumor cells undergo dedifferentiation and EMT[49] 7. MDM2/MDM4 amplification and EGFR mutation[58]	Treg cells	1. Competition with conventional T cells for IL-2 via Foxp3[66,136] 2. Secretion of the anti-inflammatory cytokines TGFβ, IL-10, and IL-35[68,69] 3. The dual expression of CD39 and CD73; the CTLA-4-mediated downregulation of CD80 and CD86 on the surface of APCs[71,73] 4. Production of FGL2 to suppress $CD8^+$ T cells and APCs through FcγRIIb[74,137] 5. Express PD-1 receptors 6. A spatial ecological niche dedicated to immunosuppression[76]
	T cells	1. Release the cytokines IFNγ[80], IL-17[86,87], IL-22[88,89], TNFα[90,91], and IL-6[92] 2. The combination of multiple cytokines, such as TGFβ and TNFα[80] or IFNγ and TNFα[93] 3. The binding of CD27 receptor to CD70 ligand[94]
	B cells	IgG4 competes with IgG1 to bind to Fc receptors on the surface of immune effector cells[107]
	Fc receptor	The binding of the Fc region of the anti-PD-1 antibody to the macrophage FcγR[62]

tumor-associated antigens[43]; (2) impairment of antigen processing and delivery, including the loss of human leukocyte antigen expression, failing to deliver tumor antigens to the cell surface[44]; (3) persistent upregulation of PD-L1 expression on the surface of tumor cells, which competes with ICI for binding to PD-1 receptors on the surface of $CD8^+$ T cells and inhibits the anti-tumor immune response[45]; (4) apoptotic resistance in tumor cells[46,47]; (5) induced dormancy and senescence of tumor cells[48], whereby the tumor cells are temporally controlled and lay the groundwork for future recurrence and metastasis; and (6) tumor cells undergo dedifferentiation and epithelial to mesenchymal transition (EMT)[49].

MDM2/MDM4 amplification and EGFR mutation

In 2017, Kato *et al.*[12] evaluated 155 patients with advanced tumors and found a 3.9% incidence of HPD. Through Next-Generation Sequencing (NGS), murine double minute 2/4 (MDM2/MDM4) amplification was identified in six patients who had TTF < 2 months and two patients were diagnosed with HPD; in 10 other patients, epidermal growth factor receptor (EGFR) mutations were identified. By multivariate analysis, it was found that MDM2/MDM4 amplification and EGFR mutations were associated with TTF < 2 months. The presence of MDM2 amplification and EGFR mutations in patients with HPD were also found in a clinical study by Singavi *et al.*[50] and Economopoulou *et al.*[51]. The MDM2 protein encoded by the *MDM2* gene is a major negative regulator of the p53 protein. MDM2 can ligate to the p53 protein through the E3 ubiquitin ligase, and the ubiquitinated p53 can be transferred to the cytoplasm and targeted for degradation by the proteasome[52]. Thus, MDM2 amplification can promote tumorigenesis directly or indirectly through the inhibition of p53. In 2018, Kato *et al.*[53] extended the scope of NGS sequencing to include 102,878 patients with different malignancies and found MDM2 amplification in 3.5% of patients; this was present in a small proportion of patients in most tumor types, and 97.6% of these patients had potentially targetable genomic co-alterations, which suggested that appropriately targeted drugs could be designed to target MDM2 amplification-induced HPD. Fang *et al.*[54] conducted preclinical studies using the MDM2 inhibitor, APG-115. It acts as an indirect p53 activator, suppresses M2 macrophage polarization, and slows tumor invasion and progression, improving anti-tumor immunity to anti-PD-1 treatment. APG-115-mediated p53 activation promoted anti-tumor immunity in TME regardless of the Trp53 status of the tumor itself. Sahin *et al.*[55] also used the MDM2 inhibitor AMG-232 in combination with anti-PD-1 antibody therapy to enhance T cell-mediated killing of tumors regardless of PD-L1 expression. Another MDM2 inhibitor, idasanutlin (RG7388), in combination with cytarabine therapy, is the first to enter phase III clinical trials for AML[56,57].

EGFR is the first identified member of the ErbB family and plays an important role in physiological processes, including cell growth, proliferation, and differentiation. EGFR is also involved in tumor development and immunotherapy-related resistance. A meta-analysis involving 21,047 patients from 35 randomized controlled trials indicated that patients with EGFR wild type had significantly prolonged PFS and OS after treatment with ICI, while those with EGFR mutations did not show any improvement[58]. This in part reflected the fact that EGFR mutations are a cause of ICI resistance. The TME in EGFR mutated lung adenocarcinoma was non-inflammatory; interestingly, the non-inflammatory TME had a high infiltration of $CD4^+$ Treg cells. EGFR signaling activates cJun/cJun N-terminal kinase and reduces the level of interferon regulatory factor-1; the former increases CCL22 and thereby recruits $CD4^+$ Treg cells, while the latter reduces the levels of CXCL10 and CCL5 and, in turn, induces $CD8^+$ T cell infiltration[59]. In addition, EGFR can upregulate the number of immunosuppressive receptors and induce the secretion of cytokines with immunosuppressive functions [IL-6, IL-10, and transforming growth factor (TGFβ)] from the TME, which in turn leads to ICI treatment resistance[60]. To some extent, this may explain the occurrence of HPD in patients with EGFR mutations after ICI treatment; however, the exact mechanism of induction needs to be further elucidated. Other somatic mutations and carcinogenic pathways exist in addition to MDM2 amplification and EGFR mutations. Xiong *et al.*[61] evaluated the mutational and transcriptional characteristics of tumors before and after anti-PD-1 immunotherapy in two patients who acquired HPD. Somatic mutations in recognized cancer genes, including tumor suppressor genes such as *TSC2* and *VHL*, were discovered, as well as transcriptional activation of carcinogenic pathways including IGF-1, ERK/MAPK, PI3K/AKT, and TGFβ.

Treg cells

Treg cells are important for the maintenance of the body's immune tolerance. The majority of $CD4^+$ Treg cells are produced by the thymus, which accounts for 10% of circulating $CD4^+$ T cells. The major transcription factor is Foxp3, which determines the phenotypic and functional characteristics of Treg cells[62]. In a normal organism, Treg cells negatively regulate immune cells such as effector T cells to prevent autoimmune overload, while, in tumors, Treg cells exhibit different biological functions[63]. Kang *et al.*[25] found significantly higher $FoxP3^+$ Treg cells in 74 patients with advanced NSCLC who developed HPD and significantly fewer Treg cells in non-HPD patients ($P = 0.024$). Therefore, PD-1^+ Treg cells could be an effective biomarker for the identification of HPD[64]. Previous studies have shown that high $Foxp3^+$ Treg cell infiltration in tumors is significantly associated with poorer OS[65]. Foxp3 is a transcriptional repressor of IL-2 that also isolates transcriptional activators acute myeloid leukemia 1 and nuclear factor of activated T-cells outside the nucleus, preventing Treg cells from producing IL-2[66]. However, Tregs and conventional $CD4^+$ T cells both require IL-2 to survive. As a result, Treg cells compete with conventional T cells for IL-2 via Foxp3 by boosting the expression of CD25 (IL2α), leading to the formation of a high-affinity IL-2 receptor (heterotrimeric complex (IL2Rαβγ)[67].

Treg cells secrete the anti-inflammatory cytokines TGFβ, IL-10, and IL-35 to deplete conventional T cells[68,69]. TGF, as a Th1 inhibitor, stimulates the TGFβRI/II receptor on conventional T cells to block IFNγ-induced Th1 activation by inhibiting the expression of two essential Th1 transcription factors, T-bet and IFN regulatory factor 1[70]. Indeed, IL-10^+ and IL-35^+ Treg cells account for a large proportion of tumors. Gene profiles of conventional T cells exposed to these Treg subtypes were analyzed, and it was discovered that T cells depletion was promoted by IL-35^+ Treg, but antitumor effects were inhibited by IL-10^+ Treg[68].

Treg cells, which have the dual expression of CD39 and CD73, block T cell activation by adenosine triphosphate (ATP) and generate adenosine to inhibit T cells. CD39 and CD73, respectively, hydrolyze ATP/ADP to AMP and AMP to adenosine, leading to a large enrichment of adenosine around Treg cells. Adenosine can induce actin cytoskeleton rearrangement and hence function as a chemoattractant for

dendritic cells (DCs), causing DCs to congregate towards Treg cells[71]. Then, with enhanced leukocyte function-associated antigen-1 stability and expression, Treg cells and DCs create a tight aggregate, decreasing the interaction of T cells to DCs[72]. On the other hand, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is mediated by Treg cells, resulting in the downregulation of CD80 and CD86 on the surface of antigen-presenting cells (APCs) to restrict the activation of conventional T cells[73]. However, it is uncertain whether the capacity of Treg cells to control CD80 and CD86 is simply dependent on CTLA-4 expression.

Treg cells produce fibrinogen-like protein 2 (FGL2) to suppress $CD8^+$ T cells and APCs through FcγRIIb. FGL2 is considered a signaling molecule for Treg cells as Foxp3 in Treg stimulates the expression of FGL2[74]. The major immunomodulatory effect of FGL2 is mediated via FcγRIIB in APCs. A study has demonstrated that mice lacking the FcγRIIB receptor develop autoimmune glomerulonephritis[75].

Treg cells can also express PD-1 receptors on their surface, and, although blocking the PD-1/PD-L1 axis activates T cells, Treg cells are also highly active, immune function is greatly affected, and anti-tumor immune efficacy is reduced. However, highly activated Treg cells in lymphoid organs resist newly generated anti-tumor T cells, leading to a more attenuated anti-tumor immune effect. These result in uncontrolled tumors, which may lead to HPD. In addition, Murakami *et al.*[76] reported a spatial ecological niche dedicated to immunosuppression which is formed between $CD8^+CD39^+PD\text{-}1^+$ T cells and $Foxp3^+PD\text{-}1^+$ Treg cells due to potential interactions between these cells in close proximity following PD-1 blockade in renal cancer. The shift to an immunosuppressive environment is more pronounced in metastatic foci. Anti-CD25 and PD-1 bispecific antibodies are currently used for treatment to deplete Treg cells. Subsequent treatment with anti-PD-1 antibodies may only enhance conventional T cells and $CD8^+$ T cells[77]. Alternatively, adenosine, a product of Treg cells, could be inhibited by combining the anti-PD-1 antibody with adenosine deaminase for its degradation to inosine, thereby reducing cAMP production to weaken the inhibition of conventional T cells and enhance anti-tumor immunity[62]. The possibility of interfering with the systemic immune system is considerably minimized by precisely destroying Treg cells around tumor cells.

T cells

The function of T cell adaptive immunity is to eliminate tumor cells that positively express antigens[78]. However, ICI-enhanced T cell adaptive immune response cannot completely kill tumor cells, as reported in most clinical trials. Even after ICI treatment, adaptive immunity can promote tumor growth directly or indirectly. As a consequence, some researchers believe that enhanced tumor adaptive immune response may be the root cause of HPD in tumor patients after ICI treatment[42]. T cell immune response can induce changes in gene expression of tumor cells, such as a downregulation of tumor surface antigens[79] and upregulation of other immune checkpoint ligands[45]. However, the underlying mechanism of T cell immune response leading to changes in tumor cells remains changes in tumor cells are still not clear.

The cytokine IFNγ released by T cells may explain a part of the problem. IFNγ, a common cytokine, is involved in several cellular changes, including EMT induction[80]. EMT in tumor cells is related to the upregulation of inhibitory checkpoint ligands[81], resistance to cell-mediated cytotoxicity[82], and the production of immunosuppressive effects[83]. Furthermore, IFNγ has the ability to upregulate immune checkpoint ligands[84], inducing tumor cell dormancy, apoptosis[84], and hyperplasia.

The same cytokine can play different roles in different environments, depending on the length of time it acts on tumor cells. For instance, prolonged exposure to IFNγ and low levels of the cytokine have been demonstrated to have pro-tumorigenic effects[85]. Other cytokines, such as IL-17[86,87], IL-22[88,89], tumor

necrosis factor α (TNFα)[90,91], and IL-6[92], are involved in tumor promotion. The combination of multiple cytokines may have a greater tumor-promoting effect than a single cytokine; for example, TGFβ1 leads to demethylation of PD-L1 promoter and TNFα leads to the expression of demethylated promoter and co-induces the overexpression of PD-L1[80]. IFNγ and TNFα can co-induce dormancy in tumor cells to promote carcinogenesis[93]. However, there is no clear answer as to which T cell subsets are mainly responsible for the release of these cytokines.

In addition to cytokines, some studies report that the binding of CD27 receptor to CD70 ligand can directly promote proliferation and differentiation of tumor stem cells[94] or T cell exosomes to induce EMT and lead to rapid tumor progression[95]. Many T cell subsets are involved, including $CD4^+$ T cells[96,97], $CD8^+$ T cells[98,99], Th1 cells[100], Th2 cells[101], Th17 cells[102], and Th22 cells[89]. However, the proportion and spatial distribution of tumor cells and effective infiltration of immune cells may be the watershed response of adaptive immunity when the tumor-immunity balance is broken.

Although there are few studies on non-Treg $CD4^+$ T lymphocytes, after ICI treatment, their levels may show an unexpected increase, which can contribute to the occurrence and development of HPD. A prospective study by Arasanz *et al.*[103] included 70 patients with advanced NSCLC who underwent ICI treatment. Early detection of HPD in NSCLC by monitoring T cell dynamics showed a strong expansion of highly differentiated $CD28^-CD4^+$ T lymphocytes ($CD4^+$ THD) between the first and second treatment cycles in HPD patients and a significant stratification among HPD patients, non-HDP patients, and effective patients (median 1.525, 1.000, and 0.9700, respectively, $P = 0.0007$). As a consequence, the strong expansion of $CD28^-CD4^+$ T lymphocytes in peripheral blood during the first treatment cycle could provide an early differential feature of HPD induced by ICI in the treatment of NSCLC. These studies suggest that $CD8^+$ T cells and Treg cells are involved in the occurrence and development of HPD in TME. However, several innate and adaptive immune cells may be swept into this storm.

B cells

PD-1 can also be expressed on the surface of B cells. Some studies have pointed out that anti-PD-1 antibodies can increase the activation, proliferation, and production of inflammatory cytokines in B cells[104]. However, follow-up studies show that the loss of B cells does not seem to have any effect on the efficacy of ICI treatment[105]. The reason for these differences may be due to the existence of different subsets of B cells. The balance among different B cells (resting B cells, activated B cells, Bregs, and other differentiated B cells) determines the ultimate role of B cells in tumor immunity[106]. Humoral immunity may play a role in carcinogenesis. Wang *et al.*[107] studied the distribution and mechanism of IgG4 secreted by B cells in the tumor model and found that the increase in B lymphocytes containing IgG4 in cancer tissues and the increase in IgG4 concentration in serum were highly correlated with the poor prognoses of patients with esophageal cancer. Using a mouse model, it was verified that IgG4 competes with IgG1 to bind to Fc receptors on the surface of immune effector cells and suppresses classical immune responses such as antibody-dependent cytotoxicity (ADCC), antibody-dependent phagocytosis, and complement-dependent cytotoxicity. Thus, tumor cell growth was indirectly promoted. Interestingly, nivolumab is essentially IgG4 with a stable S228P mutation and significantly promotes the growth of tumors in mice. However, there are only a few studies on the mechanism of B cells participating in HPD after ICI treatment, and these need further validation.

Fc receptor

The binding of the Fc region of the anti-PD-1 antibody to the macrophage FcγR consumes M1 macrophages and stimulates their differentiation to M2-like form. This is another clear mechanism of HPD after ICI treatment in addition to Treg cell-mediated inhibition of anti-tumor immunity leading to HPD[62]. The

antibody consists of $F(ab')_2$ segment bound to the antigen and Fc region bound to FcγR on the surface of immune cells. The binding of the Fc region of IgG antibody to macrophage FcγR triggers the ADCC effect, consumes M1 macrophages and NK cells, and reduces the anti-tumor immune effect[107,108]. Other studies have shown that many M2-PD-L1$^+$ macrophages were observed in the tumor tissues of NSCLC patients with HPD, which could deplete ICI through Fc-FcγR interaction, induce M2-like differentiation of macrophages, and secrete IL-10 to mediate the HPD occurrence[109,110]. The removal of ICI in the Fc region or knockout of FcγR on the surface of macrophages may be a potential research direction for further improvement[111].

CONCLUSION

HPD occurrence is currently a limitation of ICI treatment and represents the storm-like progression of tumors after ICI administration. The mechanism of HPD is similar to a "tug-of-war" between tumor and anti-tumor effects. Intervention through ICI breaks this balance. It leads to the occurrence of HPD if tumor cells are activated and the anti-tumor effect is inhibited. The side effects of chemotherapy cannot be ignored, although the present incidence of HPD in immune combined chemotherapy has been reduced. One day, we hope to usher in the era of "de-chemotherapy". Then, it would be necessary to face the problem of HPD due to ICI. Hence, the review provides a significant understanding of the current underlying mechanisms for HPD.

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Authors' contributions

Contributed to the conception of the study: Dong X
Wrote the manuscript: Ding P, Wen L
Helped perform the analysis with constructive discussions: Tong F, Zhang R, Huang Y
All authors reviewed and approved the final report.

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Cancer Drug Resistance

Perspective

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ONECUT2 as a key mediator of androgen receptor-independent cell growth and neuroendocrine differentiation in castration-resistant prostate cancer

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Abstract

Despite androgen dependence in a majority of castration-resistant prostate cancers, some cancer cells are independent of androgen receptor (AR) function, a feature of heterogeneity in prostate cancer. One of the aggressive variants of prostate cancer that are AR independent is neuroendocrine prostate cancer (NEPC). This manuscript will focus on the new finding of human one cut domain family member 2 (ONECUT2) transcription factor and its role in castration resistance, especially in NEPC.

Keywords: Prostate cancers, castration resistant, ONECUT2, androgen receptor-independence, neuroendocrine differentiation

INTRODUCTION

Prostate cancer is the most common cancer diagnosed in men and the second leading cause of cancer-related deaths in the United States[1]. Given its dependence on the AR axis, prostate adenocarcinoma can respond to androgen deprivation therapy (ADT) for a variable duration, but eventually, it progresses to

metastatic castration-resistant prostate cancer (mCRPC)[2]. Most recently, the newer generation of androgen receptor (AR) signaling inhibitors such as enzalutamide and apalutamide has improved outcomes in patients with mCRPC, but they inevitably develop resistance to these drugs as well. There are several proposed mechanisms of resistance to androgen deprivation or AR inhibitors, many of which are thought to be AR-dependent, including AR-activating mutations and constitutively active AR splice variants[2]. However, there are also AR-independent mechanisms, with very low or absent AR expression found in tumor cells that render ADT ineffective and are associated with neuroendocrine (NE) differentiation[2]. NEPC is an aggressive variant of prostate cancer that exhibits not only AR independence but also neuroendocrine (NE) differentiation and even distinct histological features such as small cell carcinoma instead of adenocarcinoma[2,3]. Though NEPC can rarely arise *de novo*, more commonly, it arises from adenocarcinoma in response to selection pressure for AR-independent cells from treatment with AR signaling inhibitors via lineage plasticity and NE differentiation[3,4]. Recent studies spearheaded by Rotinen *et al.*[5] and Guo *et al.*[6] showed that ONECUT2 is an important regulator of AR-mediated growth and a driver of NE differentiation in transition from adenocarcinoma to NEPC, and is being investigated as a new drug target with potential therapeutic implications. This manuscript explains how ONECUT2 leads to AR independence and NE differentiation observed in NEPC.

ONECUT2 AND PROSTATE CANCER

ONECUT is a family of transcription factors related to hepatic nuclear factor 6, which has been shown to be involved in organogenesis, cell fate, and tumorigenesis[7]. In particular, ONECUT1 plays an important role in hepatobiliary tract disease by regulating the development, differentiation, and function of hepatocytes and cholangiocytes[7]. Decreased expression of ONECUT1 and its target genes is associated with malformation of the liver, bile duct, gallbladder, and pancreas, as well as diabetes. Furthermore, ONECUT1 may also play a role in the prevention of hepatocellular carcinoma and pancreatic cancer by acting as a tumor suppressor[7]. In addition, ONECUT1 also helps regulate the development of retina and motor neuron[7].

ONECUT2 is another transcription factor that regulates cell proliferation, migration, and differentiation, which was first discovered by Jacquemin *et al.*[8] in 1999. ONECUT2 is found in various organs, including the liver, skin, brain, testis, and bladder[8]. In contrast to ONECUT1, ONECUT2 expression has been shown to be elevated in multiple different cancers, including prostate cancer[6], ovarian cancer[9], gastric cancer[10], colorectal cancer[11], hepatocellular carcinoma[12], lung adenocarcinoma[13], and neuroendocrine tumors[6]. Specifically in prostate cancer, increased ONECUT2 expression has been linked to the aggressiveness of the disease, disease progression, biochemical recurrence, and metastasis[5,6]. ONECUT2 mRNA level was found to be elevated in mCRPC prior to any treatment and was higher than in non-metastatic tumors[5]. ONECUT2 target genes are involved in the cell cycle, angiogenesis, and hypoxia, which in turn are implicated in tumor growth and metastasis in prostate cancer[6].

Both ONECUT1 and ONECUT2 are transcriptional activators of the ONECUT family with similar but distinct functions in development and pathogenesis in various organs. Similarities and differences between ONECUT1 and ONECUT 2 are outlined in Table 1.

ONECUT2 mediates AR independence in prostate cancer

Rotinen *et al.*[5] showed that ONECUT2 expression is negatively correlated to AR activity. AR activity was significantly lower in mCRPC tumors with high ONECUT2 expression, whereas in tumors with high AR activity, the ONECUT2 expression was suppressed[5]. ONECUT2 directly suppressed genes regulated by AR, including kallikrein-related peptidase 3 (KLK3)/prostate-specific antigen, kallikrein-related peptidase 2

Table 1. Characteristics of ONECUT1 and ONECUT2

	ONECUT1	ONECUT2
Location of gene	15q21.3	18q21.31
Target gene	- Liver genes, including hepatocyte nuclear factor (Hnf) - FOXA1/2 - Transthyretin gene - Glucokinase (Gck), glucose transporter 2 (Glut2) - miR-122	- Liver genes, including hepatocyte nuclear factor (Hnf) - FOXA1/2 - Transthyretin gene - Genes regulated by AR
Functions in development	- Expressed in retinal progenitor cells - Important for the development of liver, bile duct, pancreas - Associated with malformation of the hepatobiliary tract, maturity onset diabetes of the young	- Expressed in retinal progenitor cells - Melanocyte and hepatocyte differentiation
Associated cancers	- Pancreatic cancer - Hepatocellular carcinoma	- Prostate cancer - Ovarian cancer - Gastric cancer - Colorectal cancer - Hepatocellular carcinoma - Lung adenocarcinoma - Neuroendocrine tumors
Ref.	[7]	[7-13]

(KLK2), and ETS homologous factor[5]. Altogether, these findings show that ONECUT2 leads to AR independence in prostate cancer. Tumors with high ONECUT2 expression such as NEPC represent a variant group of mCRPC that is independent of AR function.

ONECUT2 promotes NE differentiation in prostate cancer

NE differentiation in prostate cancer is associated with a more aggressive phenotype, metastatic disease, and poor response to AR signaling inhibitors[3]. ONECUT2 has been shown to play a role in NE differentiation in prostate cancer. A significantly higher level of ONECUT2 expression was found in NEPC compared to adenocarcinoma, and conversely, a reduction in ONECUT2 expression was shown to decrease NE marker gene expression[5]. Deletion of TP53 and Rb1, two of the most frequently mutated genes in NEPC, was shown to increase ONECUT2 expression and promote NE plasticity in prostate adenocarcinoma[6]. ONECUT2 upregulates genes involved in NE differentiation, such as neuron-specific enolase, synaptophysin, and chromogranin A[2]. Furthermore, ONECUT2 has complex interactions with modulators of NE differentiation such as RE1-silencing transcription factor (REST), forkhead box A1 (FOXA1), and paternally expressed gene 10 (PEG10)[5,14,15]. REST is an inhibitory regulator of NE differentiation that directly suppresses ONECUT2[5]. A decrease in REST expression leads to upregulation of ONECUT2 mRNA and allows for the transition from adenocarcinoma to NEPC[5]. FOXA1, another modulator that normally inhibits NE differentiation, is suppressed by ONECUT2 during transdifferentiation of adenocarcinoma into NEPC[5]. PEG10 is different from REST and FOXA1 in that it promotes NE differentiation and is directly suppressed by AR[5,15]. An increase in ONECUT2 expression corresponds to upregulation of PEG10 and transition from adenocarcinoma to NEPC[5]. Figure 1 demonstrates the role of ONECUT2 in AR independence and NE differentiation.

ONECUT2 REGULATES HYPOXIA SIGNALING, WHICH PROMOTES NE DIFFERENTIATION IN PROSTATE CANCER

Guo *et al.*[6] demonstrated that hypoxia can induce NE differentiation and disease progression in prostate cancer, evidenced by an increase in NE marker gene expression in hypoxic conditions compared to normoxic ones. Knockdown of hypoxia-inducible factor 1α (HIF1α), a transcription factor that regulates the hypoxia signaling pathway, led to a reduction in NE marker gene expression[6]. Guo *et al.*[6] also showed that ONECUT2 is involved in regulating the hypoxia pathway, with ONECUT2 activity correlating with tumor

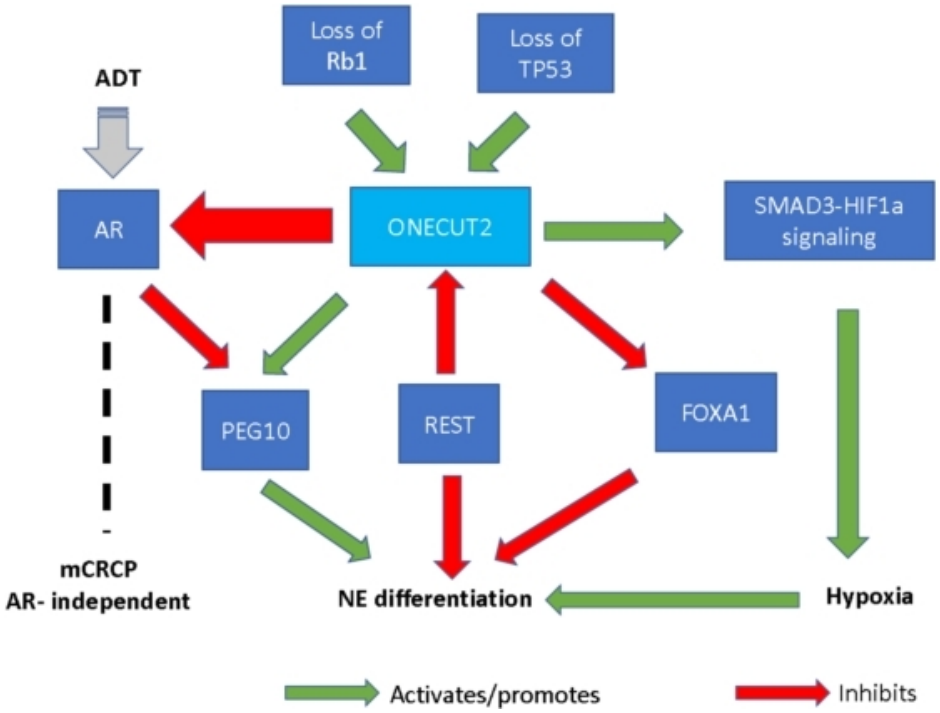

Figure 1. Role of ONECUT2 in AR independence and NE differentiation. ADT: Androgen deprivation therapy; NE: neuroendocrine; mCRPC: metastatic castration-resistant prostate cancer; ONECUT2: one cut domain family member 2; REST: RE1-silencing transcription factor; AR: androgen receptor; PEG10: paternally expressed gene 10; Rb1: retinoblastoma gene 1; FOXA1: forkhead box A1; TP53: p53-encoding gene; SMAD3: SMAD family member 3; HIF1α: hypoxia-inducible factor 1 alpha.

hypoxia in the progression from adenocarcinoma to NEPC. ONECUT2 upregulates hypoxia-associated genes such as adrenomedullin and angiopoietin-like 4 by activating SMAD family member 3, which interacts with HIF1α in regulating hypoxia signaling[6] (see Figure 1). Altogether, these findings suggest that regulating the hypoxia pathway is yet another way how ONECUT2 contributes to NE differentiation.

ONECUT2 AS A DRUG TARGET IN PROSTATE CANCER

Following the discovery of ONECUT2 and its role in the transition from prostate adenocarcinoma to NEPC, new drugs targeting this pathway are being developed and studied in clinical trials. CSRM617, a small molecule inhibitor of ONECUT2 developed by Rotinen *et al.*[5], led to cell death in prostate cancer cell lines with high ONECUT2 expression. The level of ONECUT2 expression was positively correlated with treatment response to CSRM617[5]. *In vivo*, CSRM617 led to significant reduction in tumor size, PEG10 protein level (a marker of NE differentiation), and metastatic growth[5].

TH-302 (evofosfamide) is a hypoxia-activated prodrug with an alkylating agent moiety that is released only in hypoxic environments, such as those found in hypoxic tumors like NEPC[16]. TH-302 was shown to significantly inhibit tumor growth in NEPC, but less so in adenocarcinoma[6]. There was a positive correlation between the level of ONECUT2 expression and the treatment response to TH-302[6]. These findings show that NEPC and a higher level of ONECUT2 expression are both associated with a greater degree of hypoxia, which in turn lead to an enhanced response to TH-302. TH-302 is being studied in clinical trials, including a phase I/II clinical trial (NCT00743379) that focuses on the efficacy of TH-302 in combination with chemotherapy such as docetaxel in multiple solid tumors, including mCRPC that were not previously treated with chemotherapy. Another phase I study (NCT03098160) examines the safety and

toxicity of TH-302 in combination with ipilimumab in advanced solid malignancies, including mCRPC.

FUTURE DIRECTIONS

Given the rising use of newer generation AR signaling inhibitors, mCRPC exhibiting AR independence will likely grow in prevalence[2]. Treatment of prostate cancer with AR inhibition may lead to selection for cancer cells with elevated ONECUT2 expression, thereby contributing to resistance to AR-targeted therapy and development of NE features that lead to more aggressive phenotypes with worse clinical outcomes[2,3,5]. It is, therefore, crucial to continue further research on the understanding of the master regulators of AR independence such as ONECUT2 and develop safe and efficacious drugs against such targets. We believe biomarker-driven correlative studies are key for the success of future drug development targeting ONECUT2.

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Authors' contributions

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Consent is obtained.

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Cancer Drug Resistance

Review

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Drug resistance and minimal residual disease in multiple myeloma

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Abstract

Great progress has been made in improving survival in multiple myeloma (MM) patients over the last 30 years. New drugs have been introduced and complete responses are frequently seen. However, the majority of MM patients do experience a relapse at a variable time after treatment, and ultimately the disease becomes drug-resistant following therapies. Recently, minimal residual disease (MRD) detection has been introduced in clinical trials utilizing novel therapeutic agents to measure the depth of response. MRD can be considered as a surrogate for both progression-free and overall survival. In this perspective, the persistence of a residual therapy-resistant myeloma plasma cell clone can be associated with inferior survivals. The present review gives an overview of drug resistance in MM, i.e., mutation of β5 subunit of the proteasome; upregulation of pumps of efflux; heat shock protein induction for proteasome inhibitors; downregulation of *CRBN* expression; deregulation of *IRF4* expression; mutation of *CRBN*, *IKZF1*, and *IKZF3* for immunomodulatory drugs and decreased target expression; complement protein increase; sBCMA increase; and BCMA down expression for monoclonal antibodies. Multicolor flow cytometry, or next-generation flow, and next-generation sequencing are currently the techniques available to measure MRD with sensitivity at 10^{-5}. Sustained MRD negativity is related to prolonged survival, and it is evaluated in all recent clinical trials as a surrogate of drug efficacy.

Keywords: Multiple myeloma, minimal residual disease, drug resistance, therapy, next-generation flow cytometry

INTRODUCTION

Multiple myeloma (MM) represents the second most frequent hematological malignancy, and significant survival improvements in the years have been seen[1-3]. Survival has ameliorated thanks to the availability of novel classes of drugs such as proteasome inhibitors (PIs: bortezomib, and carfilzomib), immunomodulatory drugs (IMIDs: thalidomide, lenalidomide, and pomalidomide), monoclonal antibodies (moAb: daratumumab, elotuzumab, isatuximab, and belantamab mafodotin). These new drugs are utilized alone or combined as triplets or quadruplets in the treatment of relapsed/refractory patients and thereafter at diagnosis with good results also in extramedullary MM[4-17]. MM has important clinical heterogeneity and complex genetic abnormalities[18]. Cytogenetic analysis and fluorescence *in situ* hybridization can help to distinguish different categories and risk-stratify MM patients[19]. In particular, t(4;14), t(14;20), gain of chromosome 1q, and deletion 17p give a poorer outcome, while t(11;14) and t(14;16) seem to have an intermediate prognosis[20-22]. In this view, relapses are seen in the majority of patients, and some of them still have a dismal prognosis. However, while cytogenetic analysis can help to stratify prognosis, it does not fully explain initial MM drug resistance (DR). Two main factors seem to emerge: (1) acquired drug resistance; and (2) sub-clonal heterogeneity[23,24]. Mechanisms of acquired drug resistance to the main classes of new drugs available in MM have been extensively reviewed recently[22]. A novel method to detect minimal residual disease has been developed recently[23-35], i.e., MRD detected by next-generation flow (NGF) or next-generation sequencing (NGS), which have been reported as important tools by the International Myeloma Working Group (IMWG) in the recent response guidelines[36]. MRD is an important surrogate for survival, as well as progression-free (PFS) and overall survival (OS)[37]. In particular, sustained MRD negativity confirmed at one year is of great importance to predict clinical outcome of MM patients. MRD can also detect sub-clones that acquire higher genomic instability and can ultimately drive resistance[38]. This review summarizes the main DR cell-inherent/intrinsic and extrinsic mechanisms to novel drugs in MM and will focus on recent developments regarding MRD as a tool to predict PFS and OS in clinical trials.

Drug resistance in MM

Drug resistance is the leading cause of a relapsed/refractory disease, and it can ultimately decrease survival. Many novel drugs are now available in MM with many used at diagnosis in triplet or quadruplet. MM patients can develop DR after a few cycles of therapy in a variable manner. The main mechanisms of resistance to chemotherapeutic drugs in MM can involve a drug-efflux pump, such as P-glycoprotein, or other mechanisms that inhibit the drug to enter the cell. Moreover, enzymatic inactivation of the drug or adhesion to bone marrow stromal cells, such as fibroblasts and immune cells including macrophages, in the microenvironment can be mentioned. To develop strategies for the future treatment of DR-MM patients, the main mechanisms of resistance to the different classes of drugs, namely proteasome inhibitors, immunomodulatory drugs, and monoclonal antibodies, have to be elucidated. The principal mechanisms of resistance to novel drugs and resistance escape are reported in Table 1.

Drug resistance to proteasome inhibitors

The PIs used in recent years in clinical practice are bortezomib (Bor), carfilzomib (Car), and ixazomib (Ixa)[39,40]. Bor is approved for use at diagnosis in both transplant and non-transplant eligible MM patients, while Car and Ixa are used in the relapsed and refractory treatment setting. The structure of the proteasome was first described as a hollow single-cylinder protein. The proteasome functions as an ATP-dependent organelle in which almost 90% of intracellular proteins are degraded following tagging by the polyubiquitin chain. Three subunits of the proteasome are recognized as the site of degradation in the 20S particle: β1 (caspase), β2 (trypsin), and β5 (chymotrypsin). The final result of proteasome inhibition is MM plasma cell death or apoptosis via protein accumulation[41]. Other mechanisms have been reported: p53 activation, inhibition of nuclear factor-κB (NF-κB) activity, activation of c-Jun N-terminal kinase, and stabilization of cell cycle inhibitors. PI can bind the β5 subunit reversibly (Bor and Ixa) or irreversibly (Car). Resistance to

Table 1. Principal mechanisms of drug resistance and resistance escape

C	D	M of A	DR	Resistance escape	Ref.
moAb	Dara Isa Elo Bela	ADCC, CDC, macrophage-mediated phagocytosis, apoptosis via Fc-mediated crosslinking stimulatory effects on NK cells (for anti CD38 moAb), direct cytotoxicity	Decrease target expression, complement protein increase, sBCMA increase, BCMA down expression	Change drug class, upregulation of CD38 using ATRA	[61-67]
IMIDs	Tha Lena Poma	BM microenvironment targeting; degradation of *IKZF1* and *IKZF3* via *CRBN*-dependent ubiquitination; IRF4 and *MYC* downregulation; triggering caspase 8/9-mediated apoptosis; immune modulation; anti-angiogenic activity	Downregulation of *CRBN* expression; deregulation of *IRF4* expression Mutation of *CRBN* and *IKZF1* and *IKZF3*	Change drug class, next-generation *CRBN* E3 ligase modulators (iberdomide)	[58,59]
PIs	Bort Car Ixa	Inhibition of activity of the 20S proteasome; inhibition of NF-κB activity; induction of apoptosis by activation of caspase 8/9 and p53; adhesion molecules downregulation	Mutation of β5 subunit, upregulation of pumps of efflux, HSP induction	Change drug class, pan proteasome inhibitor (marizomib), hydroxychloroquine, pan HDAC inhibitor	[46,48]

C: Class of drug; D: drug name; M of A: mechanisms of action; DR: drug resistance; moAb: monoclonal antibody; IMIDs: immunomodulatory drug; PI: proteasome inhibitors; Dara: daratumumab; Isa: isatuximab; Elo: elotuzumab; Bela: belantamab mafodotin; Tha: thalidomide; Lena: lenalidomide; Poma: pomalidomide; Bor: bortezomib; Car: carfilzomib; Ixa: ixazomib; ADCC: antibody-dependent cellular cytotoxicity; CDC: complement-dependent cytotoxicity; BCMA: b cell maturation antigen; ATRA: all trans retinoic acid; *IKZF1*: Ikaros; *IKZF3*: Aiolos; BM: bone marrow; *CRBN*: Cereblon; IRF4: interferon regulatory factor 4; NF-κB: nuclear factor-κB; HDAC: histone deacetylase; HSP: heat shock protein.

PIs is related to mutations in the β5 subunit gene (*PSMB5*) at both diagnosis and relapse[42,43]. The presence of mutations at other subunits, namely *PSMA1*, *PSMB8*, and *PSMB9*, has been described, but they have not been found to be related to resistance yet and thus need to be confirmed. Another protein important for DR seems to be the X-box protein 1 that acts in the proteostasis in the MM plasma cell. Downregulation of this pathway has been reported in PI-DR cells[44]. Decreased drug accumulation is another mechanism of DR in MM. In particular, the multidrug resistance protein P-glycoprotein (P-gp) has been well studied in the past[45]. P-gp works by implementing drug efflux from the MM plasma cell, thus reducing drug activity. Both Car and Bor have been reported to be P-gp substrates[46-48]. The mechanisms of resistance escape are reported in Table 1[49,50]. The results of preclinical studies indicate that inhibition of various heat shock proteins (HSPs), e.g., HSP90, can increase the efficacy of PIs[51]. HSPs are induced by the transcriptional blockade of protein degradation, which can contribute to drug resistance. The results from early phase I trials combining HSP90 inhibitors with PIs have identified safe doses for both drugs. In a preclinical study using bor-resistant MM cell lines, the blockade of IGF-1 downstream effectors re-sensitized cell lines to bortezomib. Furthermore, the IGF-1R inhibitor OSI-906 induced more apoptosis than Bor alone, both *in vitro* and *in vivo*[52].

Moreover, investigators used a mouse MM model to study gene-expression signatures (GES) related to Bor resistance. GES related to resistance included nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), highly expressed as part of an antioxidant-response pathway[53]. Thus, MM cells with elevated antioxidant capacity before treatment might be resistant to bortezomib.

Drug resistance to immunomodulatory drugs

The IMIDs currently used in clinical practice are thalidomide, lenalidomide, and pomalidomide (Thal, Len, and Pom, respectively). All three agents were initially used in the relapsed and refractory setting, but Len and Thal are now also approved for use at diagnosis[54]. IMIDs have antiproliferative properties and direct pro-apoptotic effects. Other mechanisms exhibited are anti-angiogenic and immunomodulatory effects, by activating NK and T cells. The E3 ligase protein Cereblon (*CRBN*) is targeted by IMIDs, in particular in complex with CRL4, ultimately leading to degradation of the Aiolos and Ikaros proteins and *IRF4* reduction[55-57]. This mechanism of action is the principal target of DR, mainly because of occurring mutations or gene expression modifications in this complex. It has been demonstrated that *CRBN* mutations appear after a relapsed/refractory disease occurs, probably due to clonal selection after long IMID therapy[58]. In addition, IRF4 has been investigated as a potential downstream target for DR in Waldenstrom macroglobulinemia after therapy with Len and Pom[59]. Methylation of *CRBN* has also been recently reported[60].

Drug resistance to monoclonal antibodies

Monoclonal antibodies directed at antigens present on the surface of plasma cells have recently entered the therapeutic armamentarium against MM. The approved moAb in clinical practice are daratumumab, elotuzumab, isatuximab, and belantamab mafodotin. Thse drugs were all tested as monotherapy first, and have since been proven to be more efficacious when combined with other agents in triplet or quadruplet[61-65].

Even though daratumumab, an IgG 1 anti-CD38 moAb, has been found to be very efficacious, resistance can be observed during treatment. Although CD38 mutations are not described as potential mechanism of DR, it has been reported that low CD38 expression could be a cause of initial resistance to therapy. Moreover, CD38 expression reduction during treatment has been described as a major mechanism and occurs via the action of sheddases[66]. Another occurring event observed is upregulation of CD55 and CD59 complement inhibitors[67]. For other moAb, the DR mechanism is not currently known, although it is logical to think that, as per daratumumab, the loss of the target antigen is likely a major mechanism of resistance[68-72].

Extrinsic mechanisms of drug resistance

The bone marrow microenvironment, including osteoblasts, osteoclasts, mesenchymal stem cells (MSC), and tumor-associated macrophages (TAM), is highly connected with MM progression and drug resistance [Figure 1]. In MM in the "osteoblastic niche", macrophages are differentiated into osteoclasts by the release of osteoclasts activating factors (i.e., IL-6, IL-1-α, TNF-α, TNF-β, and IL-11) and promote bone resorption. Osteoprotegerin represents an antiapoptotic MM factor by binding to TNF-related apoptosis-inducing ligand. Stromal cells can give drug resistance by TGF-β inhibition of osteoblasts differentiation. Stromal cells in the microenvironment have been shown to secrete several cytokines that regulate the antiapoptotic members of the Bcl-2 family (Mcl-1, Bcl-xl, and Bcl2) via IL-6 signaling[73]. A "vascular niche" is formed by endothelial cells (EC), MSC, and TAM and can protect MM plasma cells from cytotoxic drugs. In particular, EC can express an aberrant active phenotype (VEGFR-2 and FGFR-3) that can help to prevent MM apoptosis, favoring PC migration into the bloodstream and dissemination[74]. MSC can contribute to bortezomib resistance in MM via Bcl2 increased expression and enhanced NF-κB activity through cell-cell contact[74].

TAM have a basic role in MM pathogenesis, since they promote plasma cells proliferation, homing, and angiogenesis, supporting MM immune evasion and progression[75].

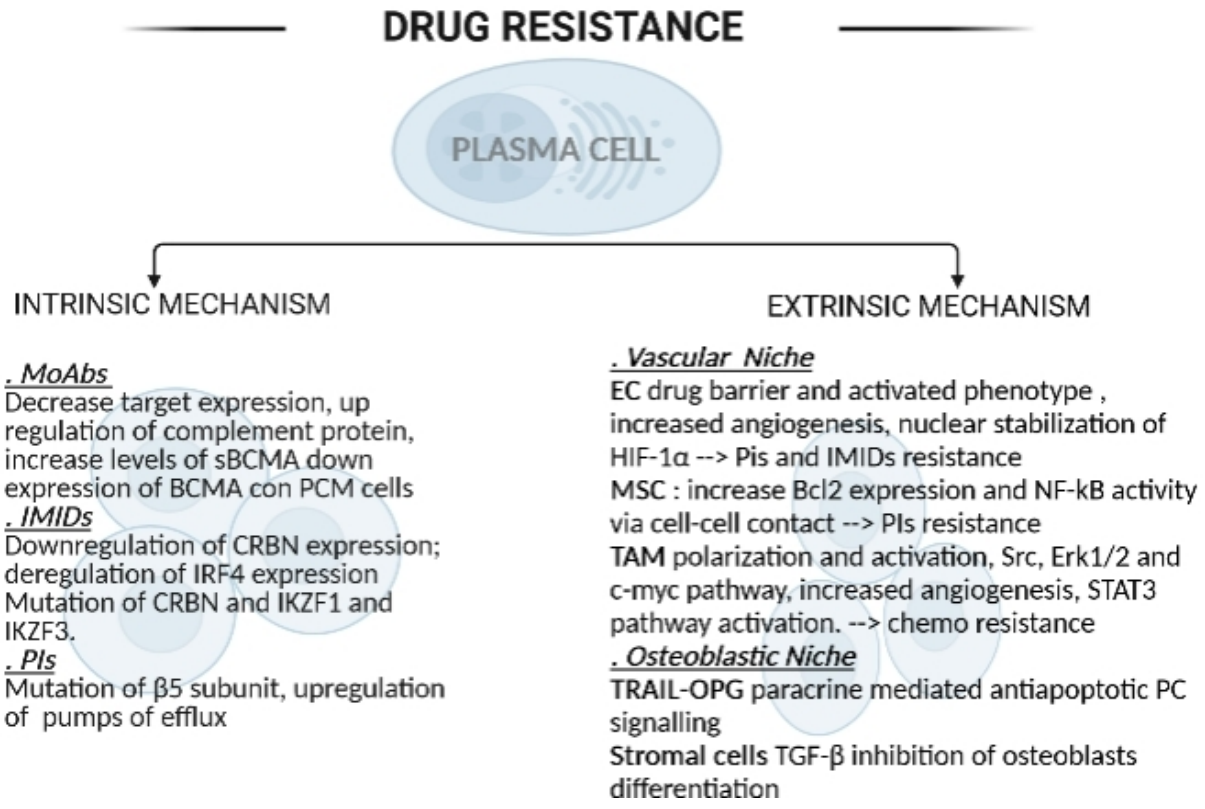

Figure 1. Drug resistance intrinsic and extrinsic mechanisms. EC: Endothelial cells; MSC: mesenchymal stem cells; TAM: tumor-associated macrophages.

MM plasma cells *in vitro* could upregulate CD206 expression and favor an M2 TAM polarization of cocultured macrophages[76]. In a preclinical model, CD68-positive TAM were shown to inhibit drug-induced apoptosis of tumor cells by caspase 3 and poly-ADP ribose polymerase cleavage[77,78]. Moreover, intracellular adhesion molecule-1 (ICAM-1) and P-selectin glycoprotein ligand-1 (PSGL-1) on the MM cells surface could activate TAM and favor TAM-induced chemoresistance through the *SRC*, *ERK1/2*, and *C-MYC* pathway[79]. VEGF production by M2 TAM was demonstrated during progression from MGUS to MM, with increased angiogenic switch[80,81].

An interesting study reported a possible correlation between the pro-tumor effect of TAM and the Stat3 pathway activation in 5T33MM cells. Interestingly, an ATP-competitive Janus kinase (*JAK*)2 inhibitor, the so-called AZD1480, could restore the sensitivity of MM cells to bortezomib[82,83].

Clinical studies confirmed CD68/CD163 double-positive M2 TAM were associated with increased microvessel density and reduced survival, independently of the MM stage[84-86]. In this field, high IHC CD163-positive M2 TAM expression at diagnosis was associated with lower complete response (CR) rate and reduced PFS and OS in 198 MM patients receiving bortezomib-based regimens[84]. Interestingly, an elevated level of soluble M2 TAM markers CD163 and CD206 was associated with worse OS; conversely, higher M1 density demonstrated a correlation with OS improvement[85].

In a retrospective study enrolling 68 MM patients, an elevated CD68-positive and CD163-positive TAM expression showed a significant reduction of six-year OS, as confirmed by multivariate analysis. As a

complementary finding, an increased CD163-positive M2 TAM number was associated with an elevated microvessel density[86]. In another relevant study, a reduced response was observed in patients presenting with high CD68-positive and CD163-positive TAM; however, only high CD163 TAM expression was related to a reduced PFS and OS. CD163 and CD168 were combined with ISS to design a new prognostic model[87]. M2 TAM infiltration and correlation with pro-angiogenic factor CD147 were investigated in a spectrum from MGUS to recurrence MM. CD163 was used as the M2 marker and the cutoff for M2 infiltration was 100 per core. The authors showed a significant OS reduction in relapsed MM patients with high M2 expression (32 months *vs.* 6 months, $P = 0.02$), suggesting a prognostic role of CD163-positive TAM in MM[88]. Finally, Andersen *et al.*[89] evaluated CD163 as a soluble marker in 104 blood samples and 17 BM samples in newly diagnosed MM patients. CD163 BM expression was higher compared to blood samples and was associated with a higher ISS stage. An elevated CD163 expression, with the suggested cutoff of 1.8 mg/L, was associated with poor prognosis, further suggesting M2 TAM could favor MM growth and progression[89].

MINIMAL RESIDUAL DISEASE

New drugs in MM have revolutionized the treatment paradigm and improved both progression-free and overall survival. However, progress in the definition of response is needed, since a CR has been defined for almost 15 years simply as the absence of a monoclonal component at immunofixation and a percentage of monoclonal plasma cells < 5% in the bone marrow.

The goal is to predict patients relapsing soon after the initial therapy and distinguish them from patients who maintain a long response (sustained CR) that is a surrogate for PFS and OS. Novel drugs are combined now in triplet or quadruplet treatment schedules and can implement in a high percentage of patients CR to a virtual disease disappearance. However, most MM patients still relapse. NGF and NGS have been introduced into clinical trials, bringing more sensitivity to detect minimal residual disease after therapy[23-34]. The detection limit of MRD is now considered 10^{-5}; however, data suggest that a deeper limit of 10^{-6} or 10^{-7} could predict a better PFS[90]. Although NGS has been reported initially to have a deeper limit of detection (10^{-6}) than NGF (10^{-5}), both can now reach comparable sensitivity at 10^{-6}.

NGF and NGS in MM

NGF has recently become part of the MRD evaluation, and it is based on the detection and quantification of normal PC *vs.* monoclonal PC using specific antibodies: PC markers such as CD38 and CD138; aberrant antigens expression such as $CD45^{-low}$, $CD19^{-}$, $CD27^{-}$, $CD81^{-}$, and CD56; CD28; CD117; and light chains κ or λ. The Euro-Flow Consortium has introduced a more sensitive standardized technique based on two eight-color tubes that permit detecting MRD with 100% applicability[24] with a 4 h sample processing time. Sensitivity needs to be at least 10^{-5}, but it has been reported as even better. A correct and adequate concentrate sample is needed to avoid hemodilution, and strategies to overcome CD38 monoclonal antibody interference have been described[26].

NGS analyzes the clonal rearrangements of the immunoglobulin heavy chain (IgH) regions with parallel sequencing of reads with a sensitivity of 10^{-6}. Importantly, patient primer construction is not needed, and the depth of detection is a strength[91]. Commercial kits are now available and FDA approved (LymphoSIGHT® and ImmunoSEQ, Adaptive Biotechnologies). Costs and a bioinformatic database for analysis as well as hemodilution are the limits[26]. NGF and NGS are recognized as complementary techniques, and the IMWG suggests utilizing the one available at the centers in clinical trials[36]. The Cassiopeia trial showed comparable sensitivity for NGF and NGS[13].

Minimal residual disease sub-clone characterization

MRD can be a surrogate for PFS and OS and is becoming an important tool for risk-stratification since the depth of response is correlated with prolonged survival[92]. In fact, a non-sustained CR can frequently be seen in MRD-positive MM patients. A goal in actual MM therapy could be to reach a sustained CR with MRD negativity lasting for at least one year. Searching for a sub-clone in MRD analysis could be an ideal way to identify drug resistance. It has been reported that MRD MM sub-clones could overexpress CD11a, CD11c, CD29, CD44, CD49d CD49e, CD54, CD138, CXCR4, and HLADR in the GEM2010MAS65 study involving 40 elderly patients[93] treated with nine cycles of VMP (bortezomib, melphalan, and prednisone) or alternating VMP to Rd (lenalidomide and dexamethasone). In particular, integrins, chemokines, and adhesion molecules were overexpressed. These chemoresistant clones with a specific multi-flow signature also displayed genetic copy number alterations (CNA) that were present since diagnosis, and the resistant clone was selected after therapy resistance. The same group recently studied a larger number of patients (n = 390) in the PETHEMA/GEM2012MENOS65 protocol (six induction cycles with Bor, Lena, and dexamethasone followed by autologous stem cell transplant, two consolidation cycles with Bor, Lena, and dexamethasone, and then randomization to maintenance therapy with Lena and dexamethasone *vs*. Ixa, Lena, and dexamethasone)[88]. They used NGF to identify detectable MRD and mechanisms related to resistance in 90 patients with high-risk (HR; i.e., del17p, t4;14, or t14;16) cytogenetics and 300 patients with standard-risk cytogenetics (SR; i.e., other anomalies not HR). Importantly, the results show the superiority of 90% PFS in MRD-negative patients *vs*. MRD-positive patients, irrespective of the cytogenetic status. Moreover, NGF studies and whole-genome sequencing showed clonal selection and higher genomic aberrations and mutations in 40 patients with multi-resistant clones[94]. Most mutations affected *KRAS*, *BRAF*, *CCND1*, *ROS1*, *NRAS*, and *FLT3* genes. CNA and mutations present at diagnosis were more likely to disappear in SR-cytogenetics patients, while HR patients had novel mutations during treatment, suggesting more genomic instability. In addition, when evaluating transcriptional clones, patients with HR cytogenetics showed the expression of PRDX6 and SOD1, which were related to a worst PFS. The authors concluded that, in HR cytogenetics MM patients, MRD resistance can arise from clones that evolve transcriptionally after therapy.

Minimal residual disease negativity after current upfront therapies for transplant and non-transplant eligible myeloma patients

With the introduction of novel drugs, responses can be seen in the majority of MM patients treated at diagnosis, both transplant and non-transplant eligible, with PFS lasting up to five years [Tables 2 and 3].

It is now evident that PFS alone cannot be the main parameter to judge new drugs in clinical trials. MRD has been proven to be a good prognosticator for PFS, OS, and drug resistance[92]. In the GIMEMA-MMY-3006 study, VTD was compared to TD in 480 MM transplant eligible patients for induction therapy and double ASCT followed by consolidation therapy[95]. VTD incorporated into a double ASCT strategy plus consolidation therapy proved to be superior to TD in terms of CR attained (58% *vs*. 41%, respectively) and PFS and OS at 10 years (34% *vs*. 17% and 60% *vs*. 46%, respectively)[96]. Although MRD was not done at that time, one study showed that, in a subset of patients treated with VTD consolidation after ASCT, MRD negativity was achieved and relapses were delayed[97]. In the IFM 2009, VRD was compared with VRD + ASCT, and in the latter group CR was superior (59% *vs*. 49%) but OS did not differ significantly[98]. Importantly, MRD negativity was significantly correlated with improved three-year PFS (87% *vs*. 42%), independently of the therapy received. Car was combined with Len and dexamethasone in the KRD regimen tested as induction and consolidation + maintenance therapy for up to 10 cycles[93,99]. Although relatively few patients were treated, MRD negativity was reached in a high percentage of patients. Recently, Dara was added to VTD and compared to VTD alone in 1085 patients in the CASSIOPEIA study[13]: response and MRD negativity were superior in Dara-VTD *vs*. VTD (64 % *vs*. 44%). Dara was added before

Table 2. CR and MRD results in transplant eligible patients with currently available therapy

Authors	Regimen	*n*	ASCT (NO/1/2)	C/M	CR (%)	MRD neg (%)	PFS/OS (months)
Cavo *et al.*[95]	TD VTD	238 236	2	C + M	41 58	NA	40.7/110 59.6/NR
Attal *et al.*[98]	VRD VRD	350 350	NO 1	C + M	49 59	65 80	37/NR 50/NR
Jasielec *et al.*[99]	KRD	76	1	C + M	78.9	70	NR
Moreau *et al.*[13]	Dara-VTD VTD	543 542	1	C + M	39 26	64 44	NR
Voorhees *et al.*[100] Laubach *et al.*[101]	Dara-VRD VRD	103 104	1	C + M	66 47	64 30	NR

PFS: Progression-free survival; OS: overall survival; CR: complete response; C + M: consolidation + maintenance; NA: not applicable; NR: not reached; ASCT: autologous stem cell transplantation; TD: thalidomide and dexamethasone; VTD: bortezomib, thalidomide, and dexamethasone; VRD: bortezomib, lenalidomide, and dexamethasone; KRD: carfilzomib, lenalidomide, and dexamethasone; Dara-VTD: daratumumab, bortezomib, thalidomide, and dexamethasone; Dara-VRD: daratumumab, bortezomib, lenalidomide, and dexamethasone.

Table 3. CR and MRD results in non-transplant eligible patients with currently available therapy

Authors	Regimen	*n*	CR (%)	MRD neg (%)	PFS/OS (months)
San Miguel *et al.*[102]	VMP MP	344 338	30 4	NA	24/56 16.6/43
Benboubker *et al.*[103]	RD cont. RD18 MPT	535 541547	15 14 9	NA	25.5/59 20.7/62 21.2/49
Facon *et al.*[12]	Dara-RD RD	368 369	48 25	31 10	NR 34/NR
Mateos *et al.*[11]	Dara-VMP VMP	356 350	46 25	28 7	36.4/NR 19.3/NR
Durie *et al.*[105]	VRD RD	235 225	24.2 12.1	NA	41/NR 29/69

PFS: Progression-free survival; OS: overall survival; CR: complete response.

ASCT to VRD in the GRIFFIN study and then compared to VRD alone[100,101]. Consolidation and maintenance therapy were also applied for up to 26 months of total therapy. MRD negativity was significantly superior in the Dara group (64% *vs.* 30%).

In the non-transplant eligible setting, great improvement with respect to the standard VMP or RD used until a few months ago[102-104] was brought recently by the addition of daratumumab. The MAIA study[12] compared Dara-Rd *vs.* Rd in MM patients not eligible for ASCT. At five years of follow-up, PFS has not been reached for the Dara-Rd group. CR was reached in 47% *vs.* 24% and MRD negativity was significantly higher (31% *vs.* 10%). The Alcyone trial[11] compared VMP *vs.* Dara-VMP. This study confirmed the superiority of the triplet with Dara, in both PFS and MRD negativity. Lastly, VRD was superior to RD in the SWOGS0777 trial in patients not proceeding to ASCT (PFS 41 months *vs.* 29 months)[105]. Daratumumab was tested as consolidation therapy in patients achieving a > VGPR after ASCT in the DART4MM study[106,107]. An interim analysis showed MRD negativity in 45% of the patients at six months of treatment. Besides the great progress achieved with the new drugs with regard to the depth of responses, MRD status should not guide clinical decisions.

CONCLUSION

MM patients still relapse after a variable period of remission. Drug resistance is a major cause of MM relapse with both intrinsic and extrinsic mechanisms. Therapeutic targeting of the bone marrow microenvironment and its interaction with MM plasma cells seems to be an ideal approach for the future. Besides the need for novel, more efficacious drugs, better prognosticators are also needed. MRD measured by NGF or NGS has entered into the MM diagnostic armamentarium and is a great prognosticator for novel drugs in clinical trials. Sustained MRD negativity is likely to be a long-term remission pre-requisite. Research has to be done to better clarify its role in identifying minimal residual sub-clones resistant to drugs commonly utilized in MM. NGF seems promising in this biological view. The characterization of these clones should be pursued in future large MM trials.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study: Gozzetti A, Pacelli P, Raspadori D, Tocci D

Wrote the manuscript: Gozzetti A, Ciofini S, Sicuranza A, Cencini E

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All authors declared that there are no conflicts of interest.

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Biological evidence of cancer stem-like cells and recurrent disease in osteosarcoma

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Abstract

Sarcomas are a large family of cancers originating in the mesenchyme. Composed of more than 100 histological subtypes, soft tissue and bone sarcomas remain clinically challenging, particularly in children and adolescents in whom sarcomas are the second most common malignant entities. Osteosarcoma is the main primary bone tumor in adolescents and young adults and is characterized by a high propensity to induce distant metastatic foci and become multi-drug resistant. The innate and acquired resistance of osteosarcoma can be explained by high histological heterogeneity and genetic/molecular diversity. In the last decade, the notion of cancer stem-like cells (CSCs) has emerged. This subset of cancer cells has been linked to drug resistance properties, recurrence of the disease, and therapeutic failure. Although CSCs remain controversial, many elements are in favor of them playing a role in the development of the drug resistance profile. The present review gives a brief overview of the most recent biological evidence of the presence of CSCs in osteosarcomas and their role in the drug resistance profile of these rare oncological entities. Their use as promising therapeutic targets is discussed.

Keywords: Cancer stem cells, bone sarcoma, soft tissue sarcoma, drug resistance, tumor microenvironment, recurrent disease, residual disease

INTRODUCTION

Sarcomas are composed of highly heterogeneous soft tissue and bone oncological entities that are members of the mesenchymal tumor family[1,2]. Osteosarcoma is the main bone sarcoma, with high prevalence in adolescents and young adults. Two peaks of incidence are described in the literature, a main peak around 18 years and a second in the sixth decade of life, more frequently diagnosed in patients following Paget's disease or radiotherapy and referred to as secondary osteosarcomas[2-4]. The conventional therapeutic regimen for osteosarcoma is based on a sequential approach combining surgery and neoadjuvant and adjuvant polychemotherapies[5]. Considered to be radioresistant, radiotherapy is nevertheless part of the therapeutic arsenal, proposed in osteosarcomas for which the surgical procedure is delicate, such as tumors in high-risk locations, and can be used for better local control of the tumor[6]. Unfortunately, the therapeutic response in osteosarcoma patients has not improved in the last four decades, with an overall survival rate of around 70% after five years for localized disease. This rate drops dramatically to 30% when lung metastases can be detected[7].

As described in other types of cancer, osteosarcoma evolves under the pressure of random mutational changes[8,9], with preferential clonal proliferation and epigenetic modifications[10-13] within the clonal population, leading to genetic instability, high genetic diversity, and high tumor heterogeneity[13,14]. Therapeutic failure is frequently attributed to this intratumoral heterogeneity, and more specifically to the emergence of oligoclonal tumor cells capable of evading the therapeutic drugs. From this observation the concept of CSCs has emerged, in reference to embryonic stem (ES) cells. CSCs express transcription factors (e.g., Nanog, Oct4, and Sox2) initially detected in ES cells and exhibit pluripotent differentiation properties into various functional cells able to reconstitute the complete tumor mass. The tumor-initiating cells, the CSCs, have been described as tumor cells capable of reproducing all features of the initial tumor mass and have been associated with tumor recurrence, propagation, and drug resistance[15-18]. Unfortunately, long-term relapse in patients considered clinically disease-free is observed in numerous cancers, including osteosarcoma[19,20]. Minimal residual disease is defined as malignant cells that are resistant to treatment and that remain in patients after remission, leading to relapse and metastasis. Minimal residual disease is composed of drug-resistant tumor cells and is presented dynamically as persister/dormant/quiescent/cancer cells in residual tumors, such as circulating tumor cells in peripheral blood and disseminated tumor cells in bone marrow and other metastatic sites[13-15,21]. In this context, tumor recurrence may be related to the persistence of CSCs. Increasing evidence highlights the existence of CSCs in osteosarcomas, although their real contribution to pathogenesis remains speculative. The present review aims to give a brief overview of the most recent knowledge available on CSCs in osteosarcoma and their potential clinical interest as new therapeutic targets.

PROPERTIES OF CANCER STEM-LIKE CELLS IN OSTEOSARCOMA AND BIOLOGICAL *IN VIVO* EVIDENCE

Around 5% of osteosarcoma patients develop local recurrence of their disease between 6 and 28 months after their first line of treatment and disease-free survival of up to 12 years is usually observed in 46% of patients[22]. A large series confirmed a relatively low rate of local recurrence of high-grade osteosarcoma in contrast to the relapse disease associated with lung metastases[23,24]. In 2010, Perrot *et al.*[20] described local recurrence with metastatic foci in patients with telangiectatic osteosarcoma of the humerus after 13 years of complete remission. The local recurrence exhibited the same histological subtype as the initial tumor and was observed at the injection site of autologous fat grafts that had been performed 18 months before the recurrence for plastic surgery. More recently, Pennati *et al.*[25] studied a series of autologous fat grafts in sarcomas and did not exclude an increased risk of local recurrence after the fat grafting procedure. These clinical cases raise the question of the persistence of cancer cells that remain quiescent at the primary tumor

site during the remission phase and are reactivated by alteration to their local microenvironment. Interestingly, in 2018, Le Nail *et al.*[26] identified osteosarcoma cells with CSC properties from high-grade osteosarcoma samples. Of the isolated cells, two showed a high ability to form spheroids, and, even though they were not tumorigenic, these cells supported tumor growth when they were co-inoculated with human osteosarcoma cell lines in immunodeficient mice.

Asymmetric cell division has been described in stable cancer cell lines, leading to the development of proliferating and quiescent cells that were functionally related to sensitive and drug resistant cells, respectively[15]. The identification of CSCs in osteosarcoma has been extensively described in the literature [Table 1]. CSCs express CD24[27], CD177[28-31], Stro-1[28-31], CD133[32-39], and ALDH1[39,41-43] and show specific metabolic properties[44-47]. Telomerase (hTert) controls the lengthening of chromosome telomeres by catalyzing the addition of repetitive DNA sequence to their end. CD271 and Stro-1 were enriched in hTert and showed metabolic specificities such an uncoupling Warburg under hypoxia[31,47]. In addition, as expected, these cells, which expressed stemness markers (e.g., *Nanog*, *OCT4*, and *Sox2*), were able to form spheroids *in vitro* and exhibited the properties of tumor-initiating cells in preclinical mouse models[47]. Among the other metabolic particularities, CSCs exhibit high aerobic glycolysis and oxidative phosphorylation[45], a downregulation of the citrate cycle, and increased oxidative glutathione levels[46] and show more generally an upregulation of most amino acid metabolisms[44,46]. A drug resistant profile has been associated with the stemness properties of CSCs, which can be modulated by epigenetic mechanisms such as DNA and mRNA methylation[48,49] and with an increase in ALDH activity and ABC transporter expression[50,51]. Interestingly, anti-cancer therapies based on cytotoxic agents result in enrichment of CSCs in cancer cells, highlighting the potentially harmful link between CSCs and the establishment of drug resistance[52-54]. CSCs may be a specific subset of tumor cells with high potential for tumor-initiation and self-renewal, as has been recently observed in all primary cultures from cases of patient-derived Ewing sarcoma[55].

MOLECULAR REGULATION OF CANCER STEM-LIKE CELLS IN OSTEOSARCOMA

Osteosarcoma growth and the distant dissemination of cancer cells are controlled by a permanent dialog between cancer cells and their microenvironment[2,56]. These soluble and membranous mediators trigger specific intracellular molecular cascades that lead to control of cellular processes, including cell death, epithelial-mesenchymal transition, or spreading, but also proliferation and quiescence. In this context, the behavior of CSCs is controlled by the tumor microenvironment. In recent decades, key signaling pathways regulating CSCs have been identified and become the source of therapeutic development [Figure 1].

The **Wnt/β-catenin** pathway contributes to the regulation of numerous cellular processes (e.g., proliferation, differentiation, and polarization) and is thus strongly associated with embryonic development. The Wnt glycoprotein family is composed of 19 secreted members that interact with cell membranes after binding to 1 of the 10 Frizzled receptors identified which are G protein-coupled receptors or to a co-receptor such as LRP-5 or -6 or tyrosine kinase receptor chains including retinoic acid-related orphan receptor and RyK. In the absence of Wnt ligand, β-catenin is degraded by the proteasome after sequestration associated GSK-3β, and the Wnt/β-catenin pathway is considered as inactive. The Wnt/β-catenin pathway is activated by the binding of one Wnt ligand to its receptor/co-receptor complex that leads to a series of phosphorylation cascades and recruitment of the receptor chains and then to the inactivation of the β-catenin degradation process. Consequently, β-catenin accumulates to the cytoplasm and is translocated into the nucleus before interacting with transcription factors, members of the TCF/LEF family, and activating target genes [Figure 1]. Any disturbance (e.g., mutations or activation) in this molecular pathway leads to pathological situations[57]. Recently, Deng *et al.*[58] studied the involvement of Indian

Table 1. Biological characteristics and functional properties of CSCs identified in human osteosarcoma

Biomarkers studied	Biological properties	Models	Ref.
CD24	- Sphere formation - Expression of stemness markers (Oct4, Nanog, Sox2, BMI1) - Properties of tumor-initiating cells - Drug resistance	- MNNG-HOS, U2OS, MG-63, and OSC228 human cell lines - Primary cultures of human cancer cells	[27]
CD117, Stro-1	- Expression of stemness markers (CD133, CXCR4, Nanog, Otc4)	- K7M2 mouse cell line	[28]
	- *In vivo* properties of tumor-initiating cells	- 318-1, P932, and K7M2 mouse cell lines and KHOS and MNNG/HOS human cell lines	[29]
	- Drug resistance (ABCG2): resistance to methotrexate, cisplatin	- U2OS human cell line	[30]
		- MG63, MNN/HOS, and 143B human cell lines and patient-derived cells	[31]
CD133	- Sphere formation - Expression of stemness markers (Sox2, Oct3/4, Nanog)	- SaOS2, MG63, and U2OS human cell lines	[32]
	- Expression of ABCG2 and MDR1	- Primary cultures of human cancer cells and MG63 human cell line	[33]
	- Expression of ABCB1, ABCC2, and the metastasis-associated genes β4-integrin, ezrin, MMP-13, and CXCR4	- FFFE samples and MG63 human cell line	[34]
	- Concomitant CD133/CXCR4 expression significantly associated with lung metastasis	- SaOS2 human cell line	[35,36]
	- Expression of CD133 and ALDH1 positively associated with lymph node metastasis and distant metastasis	- FPPE samples and SaOS2, U2OS, MG63, HOS, MNNG/HOS, HuO9, and 143B human cell lines	[37]
		- FFPE samples	[38,39]
CD271	- Sphere formation - Ability for self-renewal - Resistance to DDP therapy - Overexpression of Nanog, Oct3/4, STAT3, DNA-PKcs, Bcl-2, and ABCG2 - *In vivo* tumorigenicity	- FFPE samples and U2OS, MNNG/HOS, and SaOS2 human cell lines	[40]
ALDH1	- Sphere formation - Ability for self-renewal	- FPPE samples	[39]
	- Expression of stemness markers (CD133, CXCR4, Nanog, Otc4, Sox2, KLF4)	- MG63 human cell line	[41]
	- Drug resistance	- HuO9, OS99-1, MG63, and SaOs2 human cell lines	[42]
	- *In vivo* tumorigenicity	- HOS, MG63, MHM, MNNG/HOS, OHS, and U2OS human cell lines	[43]
hTERT enrichment	- Expression of CD117 and Stro-1 - Spheroid formation	- Primary osteosarcoma cell lines (OS1-4) - MG63, MNNG/HOS, and 143B human cell lines	[31]
Metabolic properties	- Specific metabolic feature of osteosarcoma stem-like cells: amino acid, fatty acid, energy, and nucleic acid	- 143B and MG63 human cell lines	[44]
	- Involvement of the Rap1 and Ras signaling pathways in methotrexate resistance	- OS13 human cell line	[45]
	- High aerobic glycolysis and oxidative phosphorylation: association to LINB28 expression	- HOS human cell line	[46]
	- Downregulation of the citrate cycle and elevation of oxidized glutathione levels - Upregulation of most of the amino acid metabolisms - Uncoupling Warburg and stemness in $CD133^+$ cells under hypoxia	- SaoS2 human cell line	[47]
N-methyltransferase	- Sphere formation - Expression of CD133, CD44, Oct4, Sox2, Nanog, Nestin, ABCG2, and BMI-1	- MG-63 human cell line	[48]
m^6A methylome	- Multidrug resistance - Sphere formation - Overexpression of CD117, stro-1, CD113, and stemness markers (*SOX2*, *POU5F1*, *NANOG*, *KLF4*) - Upregulation of *METTL3* and *ALKBH5* and downregulation of *METTL14* and *FTO*	- MG63 human cell line	[49]

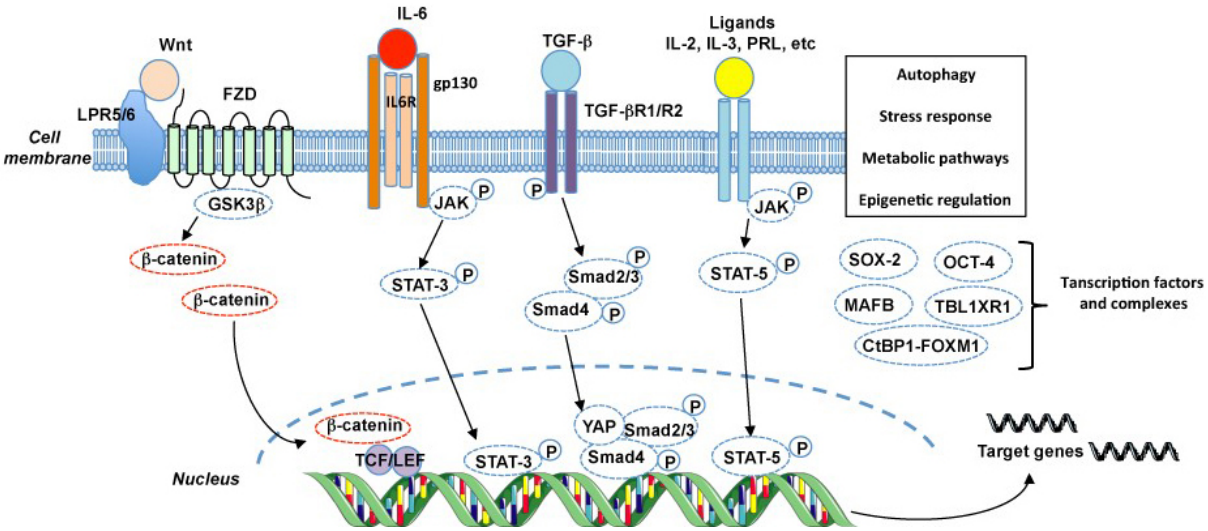

Figure 1. Main signaling pathways and mechanisms regulating the maintenance of cancer stem-like cells in osteosarcoma. LPR: Lipoprotein receptor-related protein; FZD: frizzled receptor; PRL: prolactin receptor.

hedgehog (IHH) signaling in cartilage and bone tumors by deleting *Ptch1* encoding an inhibitor of IHH receptor. They demonstrated that deleting *Ptch1* in mice was associated with an increase in Wnt member expression and the development of skeletal diseases, including osteosarcoma. Interestingly, inhibiting the Wnt/β-catenin pathway abolished the development of osteosarcoma, highlighting the key role played by this molecular pathway in the pathogenesis of bone sarcomas[58]. The Wnt/β-catenin pathway might be the link among tumor development, drug resistance, and CSCs in osteosarcoma. Whether or not the Wnt/β-catenin cascade was related to chemoresistance, it appeared to be a driver of cancer by acting directly on tumor cells, but also by modulating the immune microenvironment[59]. This cancer driver is persistently activated in the CSCs of osteosarcoma, and the stemness properties induced by chemotherapies are related to activation of the Wnt/β-catenin cascade[43,60,61]. In this context, most molecular machineries that modulate the expression level of Wnt/β-catenin may affect cancer cell behavior. Thus, epigenetic regulation of Wnt/β-catenin using the lncRNA DLX6-AS1/miR-129-5p/DLK1 axis or histone methyltransferase SETD2 results in increased stemness properties for osteosarcoma cells, tumor growth, and drug resistance[62,63]. The key contribution of Wnt/β-catenin in the maintenance of CSCs may lead to the development of new targeted therapies in osteosarcoma, as described below.

IL-6/STAT3 signaling has also been identified as a crucial regulator of bone remodeling and primary bone tumors[64]. The IL-6 family of cytokines, composed of 10 members including IL-11, OSM, and LIF, induces redundant and pleiotropic activities such as embryogenesis, differentiation, or inflammation. Most of the members of this family share the transducing receptor β-subunit gp130 as part of a multimeric receptor complex that includes a specific receptor α-subunit (e.g., IL-6R). The oligomerization of receptor subunits induced by each ligand results in various transductions of signaling pathways dominated by JAK/STAT3 activation and others such as MAPKs, p38, and JNK [Figure 1]. In addition to its functions on the tumor microenvironment (e.g., bone and immune cells), the IL-6 signaling pathway controls the maintenance of CSCs in osteosarcoma[65]. IL-6 released by activated mesenchymal stem cells (MSCs) in the tumor microenvironment promoted osteosarcoma stemness and the spreading properties of cancer cells[65]. In addition, MSCs supported drug resistance through STAT-3 signaling in cancer cells activated by IL-6[66]. MSCs and osteosarcoma cells then established a reciprocal dialog initiated by TGF-β containing extracellular vesicles secreted by cancer cells that induced the production of IL-6 by MSCs, which in turn

supported stemness, drug resistance, and tumor progression[67]. The use of active drugs confirmed the contribution of the IL-6/STAT3 axis in osteosarcoma stemness[68,69].

The TGF-β/Smad axis regulates the self-renewal of osteosarcoma cells. TGF-β belongs to a large family of at least 30 secreted proteins sharing structural similarities. TGF-β growth factors are secreted as latent precursors which can bind to specific receptor chains after activation in mature form. TGF-β induces the assembly of type I and II TGF-β receptors, leading to the formation of heteromeric receptors and the initiation of the signal transduction. The type I TGF-β receptor shows intrinsic tyrosine kinase activity, phosphorylates the type II chain, and initiates the downstream signaling, which includes Smads phosphorylation. Phospho-Smads complexes are translocated into the nucleus where they cooperate with YAP/TAZ transcription regulators and modulate the transcription of target genes [Figure 1]. Zhang *et al.*[70] studied the functional impact of TGF-β1 on osteosarcoma stemness in a hypoxic environment. They demonstrated the crucial role played by TGF-β1 on the proliferative state of cancer cells, which acquired the stem cell phenotype for self-renewal, drug resistance, neoangiogeneiss, and tumorigenicity; on the contrary, blocking the TGF-β1 signaling pathway reduced the dedifferentiation program of osteosarcoma cells. Similarly, by using gamabufotalin, a bufadienolide extracted from toad venom, it has recently been demonstrated that blockading the TGF-β/periostin/PI3K/AKT axis resulted in suppression of CSCs in osteosarcoma[71]. CSCs associated with TGF-β activity were also linked to drug resistance, as shown for EGFR inhibitors, highlighting once again the role played by CSCs in the drug resistance process[72].

Recently, **a series of transcription factors** were identified as regulators of cancer stemness in osteosarcoma. The transcription factor Sox determining the region Y-box 2 (Sox2) plays a key role in developing and controlling the embryonic stem cell state and was identified as a biomarker for CSCs in osteosarcoma [Table 1]. In addition, the proliferation of osteosarcoma cells and tumor development requires Sox2[73]. Maurizi *et al.*[73] compared tumor growth in Cre-bearing mice with identical Rb and p53 genotypes in a background of Sox2-deficient or wild-type mice. Tumor development was significantly slowed down in the Sox2-deficient mice compared to the other groups, and the survival rate was also higher in the Sox2 knockout mice. Sox2 appeared essential for the survival and proliferation of all osteosarcoma cells, including CSCs. The Hippo pathway, which is under the transcriptional control of Sox2, was directly related to the same activities, and deactivating Sox2 effectors (e.g., YAP) resulted similarly in a reduction in tumor growth[73]. Chen *et al.*[74] demonstrated that musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) is highly expressed in osteosarcoma and more specifically in CSCs, and this transcription factor, similar to Sox2, is required for the proliferation and tumorigenicity of osteosarcoma cells. Interestingly, they observed that maintaining the self-renewal potential of CSCs was under the transcriptional control of Sox-9, a stem cell regulator[74]. More recently, STAT-5 associated signaling was identified as a key regulator[75]. The knockdown of STAT-5 (A and B isoforms) using an siRNA approach reduced pimozide-induced tumor growth in mice, in addition to suppressing *in vitro* sphere formation. Inhibiting STAT-5 signaling thus impaired osteosarcoma self-renewal and development[75]. JAK/STAT-5 activation belongs to the downstream signaling associated with various cytokine/hormone-induced signaling pathways, including prolactin, IL-2, IL-3, *etc*. Oct4 promoted osteosarcoma development by supporting the maintenance of CSCs through the increase in AK055347, a long-noncoding (lnc) RNA. Oct4 knockdown with siRNA induced a significant decrease in cell proliferation, invasion, and apoptosis[76]. TBL1XR1 is a transcriptional co-factor which is overexpressed in osteosarcoma patients[77]. Its overexpression in MG63 and U2-OS cell lines induced a CSC phenotype in contrast to its repression. TBL1XR1 thus provides osteosarcoma cells with tumorigenic properties and promotes the recurrence of osteosarcoma in a STAT-3 signaling dependent manner[77]. Transcriptional complexes can also modulate osteosarcoma drug resistance. Thus, the CtBP1-FOXM1 transcriptional complex increased *MDR1* expression in osteosarcoma CSCs, which is associated

with drug resistance[78]. Interestingly, small molecules targeting this complex reversed the MDR1-mediated resistance both *in vitro* and in murine preclinical models.

Regulating osteosarcoma growth through the oct4/lncRNA axis highlights the epigenetic regulation of osteosarcoma CSCs[79]. This observation is supported by the rich literature emerging in the last 10 years[76] [Table 1]. In this context, chromodomain helicase DNA binding protein 1-like significantly reduced osteosarcoma proliferation and drug resistance though its binding to DNA. It also controls chromosomal integrity maintenance, DNA repair, and transcriptional regulation[79]. Ubiquitin-specific peptidase 39 is a crucial factor for assembling mature spliceosome complex, and its knockdown leads to the inhibition of osteosarcoma cell proliferation combined with an increase in apoptosis[80]. Human antigen R is involved in stabilizing mRNA, and its repression in osteosarcoma cells reduced their stemness properties and increased the drug response[81]. These activities were related to YAP activation. Several recent publications showed the role played by specific miRNA in controlling stemness in osteosarcoma, including miR29b and its target Spin1[82], miR34a[83] and the DNMT1/miR34a/Bcl2 axis[84,85], TNF-α-miR155 signaling[86], miR335 and its target POUF5[87], miR429 and its target Sox2[88], and the TGF-β/miR499a/SHKBP1 89 axis[89,90]. Very recently, leukemia inhibitory factor (LIF) was shown to belong to the IL-6 family of cytokines, similarly activating STAT-3, and was recently revealed as a super-enhancer-controlled regulator of CSC properties, confirming the role of STAT-3 transcription factor in the functional regulation of CSCs in osteosarcoma[91]. TSSC3 tumor-suppressing STF cDNA 3 (TSSC3), the first apoptosis-related gene reported to be imprinted, repressed the self-renewal of osteosarcoma CSCs[92]. Finally, lncRNAs also play a part in the biological regulation of CSCs in osteosarcoma[76,92,93].

Autophagy[94,95], **stress response**[96-98], and **numerous enzymatic pathways**[99-104] complete the landscape of the osteosarcoma CSC regulation mode. Autophagy was shown as a critical biological process for maintaining CSCs in OS[94], and defective autophagy was directly associated with the decrease in CSCs[95]. Similarly, the knockdown of stress-induced phosphoprotein 1 resulted in the inhibition of CSC invasiveness and migration[96]. STIP-1 is a co-chaperone that binds to HSP70 and -90 and consequently inhibits Hsp90 by 17-AAG-reduced stem cell-like properties and decreased drug resistance in OS[97].

THERAPEUTIC TARGETING OF CANCER STEM-LIKE CELLS IN OSTEOSARCOMA

The recent evidence of CSCs in osteosarcoma and better understanding of the molecular pathways required for their maintenance, led to the identification of new therapeutic targets, as summarized in Table 2.

Repressing the signaling pathways related to the maintenance of CSCs (see Table 1) resulted in the slowdown of tumor growth and inhibition of the metastatic process[105-116]. As previously mentioned, Wnt/β-catenin appeared crucial for the maintenance of CSCs and its attenuation by using tankyrase inhibitor, or tegavivint was associated with a decrease in both CSC numbers and tumor progression[105,106]. GSK3 appeared highly expressed in osteosarcoma and targeting Akt/GSK3/β-catenin or Akt/GSK3-/Notch-1, respectively, with dioscein or tideglusib repressed CSC and tumor growth[107,108]. Gamabufotalin-induced similar activities by targeting TGF-β/periostin/PI3K/Akt signaling as it has been shown for hepatocarcinoma[71,109]. Similar results were obtained by targeting BMP2R[110]. Drugs targeting transcription factors (e.g., STAT-3 and STAT5) controlling the development of CSCs may also be used to improve the therapeutic approaches to osteosarcoma[75,111,112]. Activation of hormone signaling can reduce stemness in osteosarcoma, as shown by the activation of estrogen receptor alpha by decitabine[113]. Most cytokine-induced signaling pathways result in the translocation of transcription factors which modulate the transcription of target genes. Targeting of such transcription factors (e.g., KLF4 and Sox9) may be used for reducing CSCs in osteosarcoma[114-116]. Similarly, ROCK inhibition by fasudil suppressed *in vitro* cell

Table 2. Potential therapeutic approach to CSCs in osteosarcoma

Drug	Molecular pathway involved or therapeutic approaches	Ref.
Wnt/β-catenin targeting		
Tankyrase inhibitor (IWR-1)	Attenuation of Wnt/β-catenin signaling	[105]
Tegavivint	β-catenin/transducing β-like protein 1 (TBL1) inhibition	[106]
Dioscein	Akt/GSK3/β-catenin	[107]
Tideglusib	GSK-3β/NOTCH1	[108]
TGF-β/BMP2 targeting		
Gamabufotalin	TGF-β/periostin/PI3K/AKT	[109]
BMP2	BMP2 receptor signaling	[110]
Other receptor signaling targeting (STAT-3, STAT-5, ER-α, TRAF-2, *etc.*) and transcription factors		
Bruceine D	STAT-3 inhibition	[111]
Pimozide	STAT-5 signaling	[75,112]
Decitabine	Activation of estrogen receptor alpha (ER-α)	[113]
NCB-0846	TRAF2- and NCK-interacting protein kinase	[114]
Melatonin	Suppression of SOX9 mediated signaling	[115]
Statins	KLF4	[116]
Targeting of kinase activities		
Fasudil	Rho-associated coiled-coil containing kinase (ROCK) inhibition	[100]
Autophagy and metabolic targeting		
Thioridazine	Autophagy	[94]
Metfomin	- Inhibition of mitochondrial functions (decrease in oxygen assumption, decreased mitochondrial membrane potential, decreased ATP production)	[117]
	- Pyruvate kinase isoenzyme M2 (PKM2)	[118]
	- ROS-mediated apoptosis and autophagy	[119]
	- Activation and phosphorylation of the energetic sensor AMPK	[120]
Wogonin	ROS regulation	[121]
DMAMCL	Cell cycle	[122]
DAPT	γ-secretase inhibition	[123]
Combinations with chemotherapy and sensitization to chemotherapy		
Ascorbate	Sensitization to cisplatin	[124]
Ouabain	Sensitization to cisplatin: Na^+/K^+ ATPase inhibition	[125]
Tangeretin-assisted platinum nanoparticles	Combination with doxorubucin	[126]
Senolytic drug (Fisetin)	Combination with etoposide	[127]
Immunotherapy		
Immunotherapy based on cytokine induced killer cells	CSCs spared after chemotherapy or other targeted therapies	[128,129]
Modulation of epigenetic events		
Epigenetic targeting	- USP39 silencing	[80]
	- HuR knockdown	[81]
	- Disruption of the DNMT1/miR34a/Bcl-2 axis by isovitexin	[85]
	- lncRNA HOXD-AS1 knockdown	[92]
	- RAB39A silencing	[99]
	- Targeting of lncRNA SOX2OT variant 7 by EGCG (polyphenol isolated from green tea)	[130]
Photo therapy		
- Graphene oxide nanoparticle-loaded ginsenoside Rg3	Photodynamic therapy	[131]
- CD271 antibody-functionalized HGNs	Photothermal therapy	[132]
Drug delivery systems		

- Salinomycin-loaded PLA nanoparticles	Delivery of solinomycin	[133]
- Lipid-polymer nanoparticles with CD133 aptamers	Delivery of all-trans retinoic acid	[134]
- Lipid-polymer nanoparticles with EGFR and CD133 aptamers	Delivery of salinomycin	[135]

proliferation and reduced their tumorigeneicity *in vivo*[100]. Cell metabolism is significantly modulated in CSCs (e.g., autophagy and cell cycle), and these specificities can be used for targeting CSCs in osteosarcoma. For instance, thioridazin and metformin target autophagy and metformin and wogomin modulated ROS-mediated apoptosis in CSCs and resensitize CSCs to cell death[114-116]. Similarly, regulation of cell cycle by DMAMCL or inhibition of γ-secretase by DAPT affects the behavior of CSCs and their function in tumor growth[122,123].

Drugs/effective agents can be used as sensitization agents to chemotherapy[124,125] or in combination with chemotherapeutic drugs[126,127]. Numerous cytokines are involved in the control of local immunity of cancer cells[128] and immunotherapies have been proposed for targeting CTCs[129]. Specific silencing of the epigenetic partners of CSCs can induce similar regression in tumor growth and metastatic development by altering CSC maintenance[80,81,92,95,99,129]. Nanoparticles can be used for developing phototherapies and drug delivery systems. In this context, nanoparticles have been functionalized and adapted for phototherapy with a specific aim to improve the targeting of CSCs using[131,132]. Finally, drug delivery systems have also been proposed[133-135]. For all these therapeutic approaches, the question of the general toxicity in healthy tissue stem cells and the specificity of the targeting remains unanswered.

CONCLUSION

Long considered as controversial, today CSCs are a realistic therapeutic target in osteosarcoma[1,2]. Osteosarcoma remains a highly heterogeneous oncological entity in perpetual evolution due to a strong clonal dynamic[136], leading to very efficient adaptation to drugs and the establishment of drug resistance[15]. The dynamic properties of tumor evolution have led to numerous questions about CSCs and their functional impact: (1) Can we detect CSCs in the bloodstream and can we use circulating tumor cells to follow the minimal residual disease and identify personalized therapeutic options[137]? (2) Are CSCs capable of migrating to distant organs to establish metastatic foci? (3) Is the dynamic evolution of osteosarcoma similar in the primary site and in the metastatic foci? (4) What is the functional regulation of CSCs and are they under the control of proliferating osteosarcoma cells? (5) Are CSCs regulated by the tumor microenvironment and by which molecular pathways? (6) Can we use immune therapies in combination with other drugs (e.g., chemotherapy) to target CSCs and improve overall survival in osteosarcoma? (7) How can we specifically control CSC metabolism and consequently can we set up specific therapeutic options to control CSC wake-up? (8) As osteosarcoma is a form of cancer that originates in the mesenchyme, can we use the fibrogenic reprogramming of CSCs as a therapeutic option[138]? Even whether sarcomas being considered as an immune desert explaining the current poor clinical efficacy of immune therapies needs more research[1,128], macrophage and stromal cells contribute to the establishment of drug resistance and may be identified as therapeutic target in osteosarcoma[139]. For instance, M2 macrophage may be associated with tumor angiogenesis. Tumor cells release a high number of protons that induce local acidosis, favoring the release of inflammatory mediators by local stromal cells, which in turn facilitates tumor invasiveness and metastasis in osteosarcoma[140]. Overall, it has been demonstrated that stromal cells significantly contribute to increase the stemness properties of osteosarcoma cells by inducing metabolic reprogramming of cancer cells[141,142]. Consequently, stromal cells constitute an interesting reservoir of stemness targeting to reduce osteosarcoma progression, as has been shown recently[143]. A better

understanding of the role of stromal cells in the control of stemness would help to identify new mediators associated with stemness, drug resistance, and tumor progression. Overall, CSCs are promising targets in osteosarcoma, as demonstrated by the most recent data described in this review, paving the way for a new therapeutic era focused on better-controlled residual disease in osteosarcoma through targeting CSCs.

DECLARATIONS

Authors' contributions

Supervised the work proposed and took the lead in writing the manuscript: Heymann D
Contributed to the preparation of the manuscript: Jubelin C, Muñoz-Garcia J, Cochonneau D, Moranton E, Heymann MF
All authors approved the final version submitted.

Availability of data and materials

Not applicable.

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Conflicts of interest

Jubelin C is an employee of Atlantic Bone Screen and prepared her PhD at the Université de Nantes (FR). Heymann D is a member of the Editorial board of Cancer Drug Resistance.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Emerging mechanisms of immunotherapy resistance in sarcomas

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Abstract

Sarcomas are a heterogeneous group of over 150 mesenchymal neoplasms of bone and soft tissue. Clinical prognosis remains poor in the metastatic and refractory setting, despite treatment with traditional chemotherapies. A subset of sarcoma patients can exhibit remarkable responses to novel immune therapies; however, most patients will not respond. Emerging data from genetic and transcriptomic datasets suggests that patients who are resistant to checkpoint inhibitor monotherapy may have low expression of immune-related genes, suggesting that the sarcoma was not sufficiently immunogenic to trigger or maintain an immune response to generate tumor-specific immune effector cells. In this review, we discuss the emerging data surrounding potential mechanisms of resistance, including various biomarkers explored in clinical trials of immune therapy for sarcomas. We also review future directions in clinical trials that are focused on boosting tumor immunogenicity to improve the activity of checkpoint inhibitors, as well as adoptive cellular therapy approaches to bypass deficiencies in neoantigens or antigen presentation.

Keywords: Sarcoma, immune therapy, immune resistance, immunogenicity, chemotherapy, clinical trials

INTRODUCTION

Sarcomas consist of a rare and heterogeneous group of bone and soft tissue malignancies with more than

150 different histologic subtypes by the latest World Health Organization classification[1]. While localized sarcomas can be cured with aggressive surgery and radiation treatments, in general, metastatic and refractory sarcomas are incurable with cytotoxic chemotherapies with a median overall survival of 12-18 months[2]. Sarcomas have long been theorized to be recognizable by the immune system (tumor immunogenicity), dating back to early observations by Dr. William Coley[3] in the 1890s, who noted that some patients with sarcoma had tumor regressions following bacterial infections. With the modern revolution in cancer immunotherapy, interest in exploring novel treatment approaches such as immune checkpoint inhibitors and adoptive cellular therapies has rebounded for sarcomas, with numerous clinical trials being conducted in a wide variety of sarcoma subtypes (See Table 1 for representative trials). While there have been a few promising signals of activity, such as engineered T cell therapy targeting cancer/testis antigens in synovial sarcomas, and remarkable responses to checkpoint inhibitors in alveolar soft part sarcoma (ASPS) and cutaneous angiosarcomas, responses to immunotherapy for most sarcoma subtypes have remained modest[4-10]. To further improve the activity of immune-modulating therapies in sarcomas, it is critical that we learn from the extensive work in other cancers aimed to explore mechanisms of response and resistance, and better understand the barriers existing in the sarcoma tumor and immune microenvironment. In this review, we will explore the emerging mechanisms of immune evasion in sarcoma and highlight ongoing studies in immunotherapeutics aimed to reverse this resistance.

Overview of immune response and evasion

To mount an effective immune response against cancer cells, a sequence of multiple events is required [Figure 1], with aberrations along any of the steps potentially leading to immune evasion and resistance[11]. First, the cancer cell must have immunogenic tumor-specific antigens largely resulting from genetic mutations that are processed and presented in conjunction with major histocompatibility complex class I (MHC-I). At these early phases, cancers that lack mutations (such as those driven by translocations), or present weakly immunogenic antigens, or have faulty antigen-processing machinery may fail to elicit an immune response[11]. Mutations in antigen presentation machinery components such as deletions in the beta-2 microglobulin subunit of MHC-I, deletions in the primary antigen processing enzymes such as transporter associated with antigen processing, or epigenetic silencing of MHC-I can all stall antigen presentation within the cancer cell[11]. Once antigens have been processed and peptides expressed on the surface of the cell, antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages must then respond to immunogenic cytokines from the cancer cells to be drawn into the tumor microenvironment (TME) and recognize the antigens[11]. Insults that trigger immunogenic cell death of the cancer cells, such as certain types of chemotherapy and radiation, can lead to the production of type I interferons and release of other damage and pattern-associated receptors (DAMPs and PAMPs) that improve APC migration and activation[12]. Effective APC surveillance and uptake of antigens can then lead to the priming of tumor-specific T cells in tumor-draining lymph nodes that then expand, traffic, and infiltrate into the tumor beds[11]. Alternatively, cytotoxic T cells may directly surveil MHC-I bound antigen on the surface of the cell to trigger activation and T cell-mediated killing. Regardless, at the final stages of the cytotoxic response, the cancer cell: T cell synapse is again modulated by the expression of immune checkpoint proteins such as the programmed cell death protein 1/programmed cell death protein ligand 1 (PD-1/PD-L1) axis, which can abort T cell-mediated killing even in the setting of a robust prior immune response[11].

A major obstacle for both APC and T cell trafficking and function is navigating the suppressive TME and retaining cytotoxic and anti-tumor potential under stress from faulty metabolism, aberrant angiogenesis, and stromal factors that slow or inhibit the infiltration of immune cells into the tumor bed. Both DCs and macrophages are versatile innate immune cells with functional plasticity that is influenced by the TME. Activated and suppressive phenotypes exist in a delicate balance, and with prolonged exposure to the TME,

Table 1. Selected immune checkpoint inhibitor combination clinical trials for sarcomas by mechanism of resistance

Resistance mechanism	Agents	Ref. (if data available)	NCT# (if ongoing)
Suppressive immune phenotypes (TAMs, Tregs)	Cyclophosphamide/pembrolizumab	[65]	-
	DCC-3014 (CSF1R inhibitor)/avelumab	[64,65,90]	**NCT04242238**
Microenvironment	Axitinib/Pembrolizumab	[4]	-
	Sunitinib/Nivolumab	[36]	-
	Apatinib/Camrelizumab	[37]	-
	Cabozantinib/Nivolumab +/- Ipilimumab	-	**NCT04339738** **NCT04551430** **NCT05019703**
Tumor immunogenicity	Radiation +/- pembrolizumab	-	**NCT02301039**
	Cytokines:		
	NKTR-214/nivolumab	[75]	-
	IFN-gamma/pembrolizumab	-	**NCT03063632**
	Oncolytic viruses:		
	Talimogene laherparepvec (TVEC) /pembrolizumab	[91]	-
	Chemotherapy:		
	Doxorubicin/pembrolizumab	[32,87]	-
	Doxorubicin/zalifrelimab/balstilimab	-	**NCT04028063**
	Doxorubicin/ifosfamide/PD-1 blockade	-	**NCT04356872**
		-	**NCT04606108**
	Gemcitabine/docetaxel/retifanlimab	-	**NCT04577014**
	Gemcitabine/pembrolizumab	-	**NCT03123276**
	Gemcitabine/docetaxel/doxorubicin/nivolumab	-	**NCT04535713**
	Trabectedin/ipilimumab/nivolumab		**NCT03138161**
	Eribulin/pembrolizumab	[88,89]	**NCT03899805**

there is a preponderance of suppressive immature DCs and tumor-associated macrophages (TAMs). TAMs have a complex nomenclature, with numerous functionally distinct phenotypes. A common terminology used includes M0, newly recruited monocytes to the TME prior to differentiating to M1/M2; M1, classically activated macrophages, which have anti-tumor properties; and M2, alternatively activated macrophages which have immunosuppressive activity[13,14]. Finally, T cells progressing through various activation stages will eventually become exhausted, losing their cytotoxic potential, or skew towards suppressive phenotypes such as T regulatory cells[15].

The landscape of immunotherapies being studied in sarcoma aims to reverse immune evasion by the cancer cells at each of these steps. However, the immune evasion and kill balance is a highly dynamic process that is dependent on numerous factors, including tumor heterogeneity within individual tumors even within individual patients, as well as extensive host factors[16]. A variety of modalities ranging from checkpoint inhibitors, adoptive T cell transfer, oncolytic viruses, cancer vaccines, and cytokine modulation have been tested. However, one of the biggest challenges in immunotherapy in general and particularly in sarcomas is understanding the mechanisms of resistance to these therapies at any particular time, to tailor and develop therapies that can overcome the chief resistance mechanisms sequentially, or multifaceted approaches that target multiple steps in the cascade simultaneously. We will now review the emerging data in sarcomas for resistance and how this is impacting the development and efficacy of immunotherapeutic strategies.

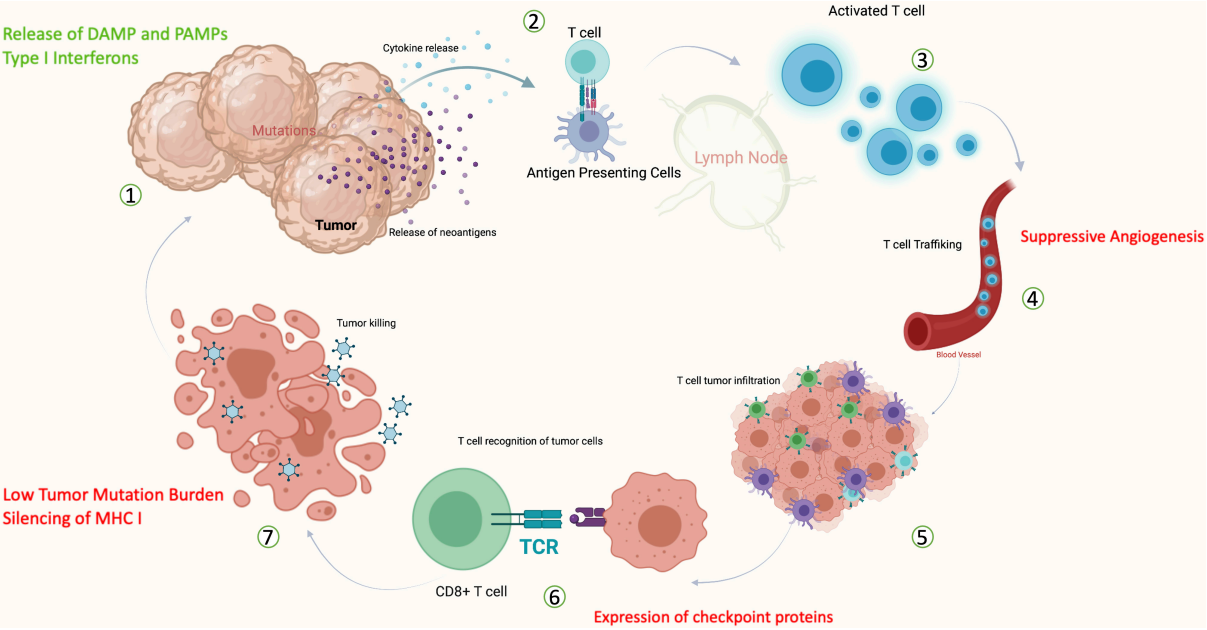

Figure 1. Multiple steps are required to generate immune response against cancer. Inhibitory factors are noted on red and facilitating factors in green. DAMP: Damage-associated molecular patterns; PAMPs: pathogens-associated molecular patterns; MHC-I: major histocompatibility complex class I; TCR: T cell receptor. Created with BioRender.com.

EMERGING MECHANISMS OF RESISTANCE

Preclinical investigations in murine models

In-depth exploration of the sarcoma immune microenvironment has been limited due to the few numbers of syngeneic murine models with a competent immune system. Additionally, those models that do exist fail to capture the heterogeneity within a large number of sarcoma subtypes, genetic and immune heterogeneity within sarcomas even of the same subtype, and inter-and intratumoral heterogeneity within the same patient. Wisdom *et al.*[17] recently reported immune profiling in a high mutational load murine model of undifferentiated pleomorphic sarcoma (UPS), generated by inducing conditional *Trp53*$^{fl/fl}$ deletions with subsequent injection of the carcinogen 3-methylcholanthrene in the same site. The authors demonstrated resistance to radiation and PD-1 blockade in primary tumors that evolved under immune pressure, compared to transplanted tumors in different mice, which were more readily rejected. As expected, transplanted tumors showed a more inflamed microenvironment with increased activity of suppressive M2 macrophages and activated CD8+ T cells. Treatment with PD-1 blockade further increased CD8+ T cell activity with a decrease in M2 macrophages, which was not recapitulated in the primary tumors[17]. However, another study performed similar experiments in the KP model, with conditional *Kras*^{G12D} mutation plus *Tp53* deletion, comparing the immune infiltrates and response to checkpoint blockade in primary tumors to those transplanted into naïve C57Bl/6 mice. Interestingly, in the setting of a low mutational burden tumor, there was no improvement in immunogenicity despite the transplantation, and despite an increase in immune cell infiltration, there was no improvement in response to checkpoint blockade[18]. Finally, a third paper summarized divergent immune landscapes with transplantation of primary KP UPS and rhabdomyosarcoma models into syngeneic recipient mice. Again, a more robust immune infiltration was seen in the recipients, which was associated with higher levels of MHC-I expression[19]. Taken together, these papers illustrate that for "cold", antigen-poor primary sarcomas, recognition of the cells as foreign is critical to mounting an effective immune response. The lack of immunogenic neoantigens or failure of the initial recognition by APCs is likely one of the central mechanisms of resistance to immunotherapy for the majority of sarcomas.

Global assessment of immunogenicity using immune-related gene expression analysis

With the increase in available transcriptomic data, multiple studies have recently investigated immune-related gene expression signatures in human sarcomas. In an analysis of 213 sarcoma tissue samples from The Cancer Genome Atlas (TCGA), including leiomyosarcoma (LMS), UPS, and dedifferentiated liposarcomas (dLPS), deconvolution analysis showed that the majority of sarcomas lacked high expression of immune-related pathway signatures. However, 20% exhibited an immune-high expression signature which correlated with responses to pembrolizumab monotherapy and was characterized by the presence of tertiary lymphoid structures (TLSs), comprised of CD8+ T cells, B cells, and follicular dendritic cells[20]. Additionally, these sarcomas showed high expression of early immune response signatures, including MHC-I. Close to 80% of the sarcomas had a low or heterogeneous immune signature expression, supporting clinical observations of checkpoint inhibitors inducing a response in only about 20% of patients[4-10]. A subsequent paper utilized the CIBERSORT algorithm to query microarray expression data in 253 soft tissue sarcomas [synovial sarcoma, myxoid liposarcoma, non-translocation sarcomas with more complex genomic profiles, and gastrointestinal stromal tumor (GIST)] and identified immune signatures that were largely clustered within the histologic subtype. Several potential targets of interest were identified for the various histologies, such as CD40 in GIST and synovial sarcomas, and M0 macrophages were enriched for all subtypes. These signatures had some prognostic implications but were not correlated with any prior immunotherapy exposure[13]. A third paper from Hu *et al.*[21] similarly reviewed immune-related expression signatures in 259 patients with soft tissue sarcomas and again demonstrated four unique clusters of immune-related genetic expression and demonstrated associations with prognosis, including overall survival and progression-free survival. The worst prognosis cluster had the lowest immune score, lowest stromal score, and lower expression of a variety of checkpoint proteins. These collective investigations again support that failure to generate an immune response, evidenced by a lack of downstream immune activity, may be central to the resistance of most sarcomas to immune targeting therapies.

Neoantigens and tumor mutational burden

It has been widely recognized that a high tumor mutational burden (TMB), which can be detected by whole-genome sequencing (WGS), can lead to increased neoantigen expression and can correlate with immunotherapy responses[22,23]. Although TMB is not a perfect biomarker, most sarcomas do have a lower TMB than the currently approved tissue agnostic threshold (10 mutations/Mb) that has been shown to predict responses to checkpoint inhibitors[24]. However, 13.7% of angiosarcomas, 8.1% of UPS, and 8.2% of malignant peripheral nerve sheath tumors (MPNSTs) were found to have more than 20 mutations/Mb in an analysis of 100,000 different cancers[24], potentially explaining the differential activity of immune checkpoint inhibitors (ICIs) in these subtypes. In contrast, in subtypes where responses to ICIs are poor, like synovial sarcoma and osteosarcoma, only 1% and 0.4% respectively had high TMB greater than 20 mutations/Mb[24]. However, TMB does not fully explain the immunogenicity of individual sarcomas with low TMB or for remarkable responses seen in translocation-associated sarcomas such as ASPS[25]. Some evidence exists that perhaps genetic abnormalities not picked up with standard WGS, such as fusions without an RNA product, or indels, may help to drive responses. We previously reported whole-exome and RNA sequencing results for a patient with cutaneous angiosarcoma who had a complete response to very low dose cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibition on a phase 1 clinical trial[5]. While many cutaneous angiosarcomas have been shown to have high TMB with an ultraviolet radiation damage signature resembling melanoma[26], our patient had a very low TMB at 0.09 mutations/Mb. However, numerous putative fusion transcriptions were identified, including 31 fusions predicted to generate novel protein sequences. Increasingly, epitopes resulting from non-traditional genetic alterations, such as indels, copy number alterations, or single nucleotide variants, have been recognized to be potent antigens, potentially driving responses to immunotherapy[27-29]. As shown in other cancers, the quality of an antigen may dominate over the number of predicted antigens, which may not be conserved from patient to patient, and

is dependent on the unique characteristics of the individual patient's MHC alleles.

Finally, there is emerging evidence that particular genetic alterations may profoundly impact oncologic signaling pathways and the development of resistance to immunotherapy. One powerful example is a patient with metastatic leiomyosarcoma who achieved a durable response to checkpoint blockade for more than two years, but then developed oligoprogression in a single metastatic site. Genetic profiling revealed loss of *PTEN* in the progressing lesion, which reduced neoantigen expression and resulting immunogenicity[30]. Another well-reported biomarker of immunotherapy resistance is *MDM2* amplification which has been linked with hyperprogressive disease with ICIs[31]. However, liposarcomas that are defined by *MDM2* amplification can respond to ICIs alone or in combination with chemotherapy[9,32]. Further analysis of genetic mutations in correlation with TME features, such as PD-L1 expression and phenotyping of infiltrating lymphocytes, will be critical to better understand the impact of these genetic aberrations on the sarcoma immune microenvironment.

Tumor microenvironment

Another leading hypothesis for resistance to immune therapies for sarcomas lies in the hostile TME. Sarcomas tend to be large with aberrant angiogenesis, with frequent overexpression of major suppressive cytokines such as vascular endothelial growth factor (VEGF)[33] or transforming growth factor beta 1 (TGFB1)[34]. Since sarcomas are, by definition, stromal/mesenchymal neoplasms, many have theorized that suppressive forces from the stroma itself may drive resistance to immune therapy.

VEGF has been implicated in numerous complex mechanisms of resistance, including the promotion of immature and suppressive phenotypes of DCs, TAMs, and T regulatory cells, impairment of T cell trafficking and migration via faulty angiogenesis, and altering of various physiologic processes via maintenance of hypoxia[35]. The activity of various tyrosine kinase inhibitors (TKIs) with anti-VEGF activity has led to multiple combination studies with checkpoint blockade in both bone and soft tissue sarcomas[4,36,37]. While the mechanisms through which angiogenesis growth factors enhance immunotolerance could be related to increased vascular permeability[33], correlative investigations to identify biomarkers of response and to elucidate resistance to combination anti-VEGF TKI therapy are still under investigation and will be discussed later in this review.

TGFB has also been implicated as a global mediator of resistance to cancer treatment, including immunotherapy[38]. TGFB has been shown to directly inhibit T cell function by promoting FOXP3 expression on T cells, impairing natural killer cell function, decreasing MHC class II expression, and promoting suppressive phenotypes of TAMs and neutrophils[38]. While the role of TGFB is underexplored in sarcomas, several studies have shown that TGFB-mediated expression of epithelial-mesenchymal transition (EMT)-type transcription factors, including Snails and Twist, promotes invasion and metastasis of osteosarcoma cells[39]. Clinical investigations of TGFB inhibition in sarcoma are very limited, with the inclusion of only a few patients in all-comer Phase 1 studies[40]. However, Vigil is an autologous tumor cell product where cells are transfected with a plasmid containing the stimulatory cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), and a short hairpin RNA to knock down furin, an enzyme required for the production of TGFB1 and TGFB2. Initial results of Vigil in patients with Ewing's sarcoma showed promising results, with a 73% 1-year survival for Vigil-treated patients compared to 23% in a contemporaneous cohort of patients who did not receive Vigil[41]. An ongoing Phase 3 clinical trial of Vigil with irinotecan/temozolomide (NCT03495921) aims to confirm the benefit of targeting TGFB in this population, and may support further investigation of targeting this mediator.

An emerging suppressive factor of interest is sialoglycans, which are sialic acid sugar-carrying glycans, and are suggested to mediate regulatory pro-tumor growth functions of the TME and to induce angiogenesis regulated by macrophages[42,43]. Sialoglycans are recognized by sialic acid-binding immunoglobulin-like lectins (Siglecs), a family of immunomodulatory receptors that are expressed predominantly on immune cells. Tumor sialoglycans can interact with the Siglecs and may modulate the immune TME and stimulate further Siglec expression on the infiltrating immune cells[44]. Each Siglec preferentially recognizes a different type of sialic acid. Siglec-15, in particular, is selectively expressed in myeloid cells and osteoclasts[45]. While studies in other types of sarcoma are lacking, preclinical studies on osteosarcoma cell lines and xenograft mouse models discovered that downregulation of Siglec-15 led to decreased tumor proliferation by inducing EMT and promotion of DUSP1[46]. Following that finding, an analysis of 36 human osteosarcoma samples also showed that higher expression of Siglec-15 by immunohistochemistry was associated with worse overall survival compared with tumors with low Siglec-15 expression[46]. A pan-cancer analysis utilizing the TCGA database, which included sarcoma, showed that upregulation of Siglec-15 correlated with shorter overall survival and progression-free survival (PFS) in the sarcoma cohort[47]. Siglec-15 also positively correlated with T-regulatory cells and macrophages in most cancers[47]. Thus, it is intriguing to speculate that the negative impact on survival from Siglec-15 expression could result from its immunosuppressive activity in sarcomas.

Biomarkers of resistance identified in clinical explorations of immunotherapy vaccines

With a central mechanism of resistance in sarcomas appearing to revolve around a lack of neoantigens that can trigger antigen presentation, immune recognition, and adaptive immune responses, it is not surprising that much hope has previously been placed in the use of therapeutic vaccines. Despite extensive efforts, the development of therapeutic vaccines in sarcomas has shown limited efficacy thus far. For example, many pediatric sarcomas express the gangliosides GM2, GD2, and GD3[48], leading to a study of a peptide vaccine targeting these markers in 136 patients with a variety of sarcomas. Despite high expression, the vaccine did not show any benefits in survival despite eliciting a sustained serologic response[49]. In other attempts, dendritic cell vaccines were developed using patient-derived dendritic cells loaded with tumor antigens *ex vivo*. Despite the notable serologic response, this strategy has also been unsuccessful; only 1 out of 35 evaluable patients had a partial response[50]. The lack of response to vaccines has been attributed to downstream mechanisms of immune suppression, possibly secondary to increased tumor burden[50]. Vaccines using peptide-pulse dendritic cells with a tumor antigen have also been used in an attempt to avoid surgical resection of tumors required to isolate tumor lysates. This strategy was also disappointing[51] with no long-lasting responses secondary to the short lifespan of the cells in the circulation. Overall, vaccines are likely to require combination approaches, potentially agents targeting the microenvironment and/or checkpoint blockade to further improve efficacy.

Immune checkpoint inhibitors

Immune checkpoint inhibitors have transformed the treatment of multiple solid and hematologic malignancies since their initial success in melanoma and non-small cell lung adenocarcinoma. The antibodies currently approved target the inhibitory proteins PD-1, PD-L1, and CTLA-4, and block the suppressive signals towards T cells in the TME. In sarcomas, the success of checkpoint inhibitors alone is limited for most subtypes, with responses ranging consistently less than 20%[9,52]. Exceptions to those responses have been observed in a few histologies such as UPS, dLPS, ASPS, and cutaneous angiosarcoma[4,5,9,52]. Since ICIs are now the most commonly tested immunotherapy strategy for sarcoma patients, the majority of our knowledge on immunotherapy resistance mechanisms stems from these trial results.

The expression of PD-L1 by cancer cells is an important predictive factor of response to ICIs in many cancer types and is used as a biomarker of favorable results[53]. However, in sarcomas, the predictive value of PD-L1 expression for responses to ICIs as well as outcomes has been inconsistent. Overall though, most subtypes show consistently low expression of PD-L1 in the range of 10%-22%[54-57] and higher in certain subtypes such as UPS and ASPS[4].

There is some evidence that the presence of tumor-infiltrating lymphocytes (TILs) and other immune cells within the sarcoma TME may be more predictive of ICI responses compared to PD-L1 expression on cancer cells. The prevalence of TILs in most common sarcoma subtypes has been reported to be as high as 98%[58,59]; however, some of these studies are limited in phenotyping to determine the function of visualized cells. It has been observed that tumors that lack TILs are unlikely to respond to ICIs, regardless of PD-L1 expression[60]. In the SARC028 trial of pembrolizumab in patients with advanced sarcoma, the objective response rate was only 17.5%[9]. Patients who responded to ICIs, albeit few, had significantly higher infiltration of T cells (CD8+, CD3+, PD-1+) and TAMs that were PD-L1 positive at baseline compared to nonresponders[61]. Across a variety of numerous soft tissue sarcoma subtypes, UPS was found to have a higher density of TILs with more oligoclonality compared to other subtypes, further highlighting the role of TILs in ICIs responses[57]. However, ASPS patients treated with axitinib/pembrolizumab did not show higher TIL infiltration by immunohistochemistry compared to non-ASPS patients, but had universally positive PD-L1 expression, despite the higher response rate[4].

Taking T cell infiltration one step further, TLSs are organized lymphoid aggregates containing CD8+ T cells, B cells, and follicular dendritic cells[20], that influence anti-tumor immunity in cancers, well-described in melanoma and renal cell carcinoma[62]. TLSs have been identified in various sarcoma subtypes, including UPS, dLPS, LMS, and rhabdomyosarcomas[20,63], and their presence correlated with response to pembrolizumab monotherapy[20]. As such, TLSs may offer a surrogate of an organized and robust anti-tumor immune response and may be used as a biomarker of response to immunotherapy, as explored in a dedicated arm in a larger phase II clinical trial of pembrolizumab and cyclophosphamide in soft tissue sarcomas[64]. Out of 240 sarcoma patients screened for the TLS-containing cohort, 35 evaluable TLS-positive sarcoma patients achieved a 30% objective response rate, with 33.3% achieving stable disease. The overall response rate for the unselected patient population in this trial was only 2%[65].

In addition, many soft tissue sarcomas and GIST tumors have a predominant expression of M2 macrophages, the immune-suppressive phenotype[65]. Additionally, myeloid-derived suppressor cells (MDSCs), which are regulatory cells promoting tumor growth and immune evasion in the TME, have been shown to correlate with resistance to ICI in patients with melanoma[66]. In sarcoma, MDSCs have been reported to correlate with higher stage and metastatic tumor burden, possibly supporting their immunosuppressive role in the sarcoma TME[67]. Further evidence to support that was the findings of enhanced responses with ICI treatment in a murine model of rhabdomyosarcoma upon inhibition of the MDSCs trafficking to the TME[68].

Adoptive cellular therapy

Adoptive cellular therapy aims to bypass the priming and activation of the T cells by providing *ex vivo* activated T cells against previously-identified tumor antigens directly to the patient. Three adoptive cellular therapies under study currently in sarcomas include engineered T cell receptors (TCR), which recognize MHC-I-restricted antigens, Chimeric Antigen Receptor (CAR) T cells which can recognize cell surface antigens, and adoptive transfer of TIL[69]. Cellular therapies harnessing engineered TCRs or CARs require antigen recognition in the tumor, preferentially expressed by tumor cells and not by normal cells. New

York-esophageal squamous cell carcinoma-1 (NY-ESO-1) and melanoma-associated antigen-A4 (MAGE-A4) are two unique cancer/testis antigens expressed in selected sarcoma subtypes. NY-ESO-1 expression has been observed more frequently in synovial sarcomas (> 60%) and in myxoid liposarcomas (88.2%) and less frequently in pleiomorphic liposarcoma (21.4%), chondrosarcomas (14.3%), osteosarcomas (31.3%), and desmoplastic small round cell tumors (16.7%)[70,71]. Similarly, MAGE-A4 has been detected more commonly in synovial sarcomas (82.2%), myxoid liposarcomas (67.7%), osteosarcomas (43.8%), angiosarcomas (41.4%), MPNSTs (24.6,%) and chondrosarcomas (21.4%)[70,71].

The responses to engineered T cells specific to NY-ESO-1 in synovial sarcomas are definitely encouraging, with a 50% objective response rate in the first cohort of treated patients[8]. A major challenge for TCR-directed therapies, though, is the required human-leukocyte antigen (HLA) compatibility, with currently studied agents restricted to HLA-A*02 subtypes. In the first cohort of patients treated with NY-ESO-1 T cells, 120 patients were screened for HLA subtype and antigen expression to enroll 15 patients[8]. An important insight from this study was that contrary to other cancers, loss of antigen expression and loss or mutations of genes mediating antigen presentation, including MHC-I, were not associated with acquired resistance in patients with synovial sarcoma treated with SPEAR T cells targeting NY-ESO-1[72]. However, the lymphodepleting chemotherapy regimen is important for the effectiveness of the TCR therapies in sarcomas; it favors the combination of fludarabine and cyclophosphamide and maximizes the engraftment of adoptively transferred T cells via IL-7 and IL-15[72].

CAR T cells provide an alternative to the HLA compatibility challenge that limits TCR therapy. However, they require antigens expressed on the surface of the tumor cells rather than peptide-MHC complexes (intracellular antigens). Extracellular antigens are limited in sarcomas overall, with a few exceptions of GD2 in osteosarcoma, rhabdomyosarcoma, and Ewing's sarcoma; platelet-derived growth factor receptor α in rhabdomyosarcoma and HER-2 in osteosarcoma[48,73,74]. Studies are currently underway to assess the safety and efficacy of these and other therapies, including CAR T cells directed at B7-H3 (NCT04897321), EGFR (NCT03618381), and GD2 (NCT02107963, NCT04539366, NCT03721068, NCT03635632).

Finally, TIL therapy is under early investigation in sarcomas; in this modality, autologous TILs are collected from an individual patient's tumor and then expanded, and then returned to the patient with lymphodepleting chemotherapy and interleukin-2 (IL-2) therapy. An ongoing clinical trial is exploring the safety and efficacy of this approach (NCT04052334).

Overcoming resistance: future directions

While immune therapies, including checkpoint inhibitors and adoptive cellular therapies, have had promising activity in a subset of sarcoma patients, it is clear that other strategies will be needed to overcome innate resistance to immune-mediated recognition and kill the majority of sarcomas. Over the past several years, various trials have tested combination therapies aimed at reversing resistance mechanisms at various steps in the cascade, including targeting the suppressive immune microenvironment with metronomic cyclophosphamide[65], or with VEGF blockade[4,36,37]. Selected trials representing the scope of previous and ongoing strategies to overcome key potential mechanisms of resistance discussed in this review are listed in Table 1.

While combination VEGF blockade and checkpoint inhibitors have shown activity in ASPS[4] and osteosarcoma[37], outcomes in other soft tissue sarcomas have not been convincingly different from what could be expected from checkpoint inhibitor therapy alone. Ongoing trials are assessing the activity of broader-spectrum anti-VEGF TKIs, such as cabozantinib (NCT04339738, NCT04551430, NCT05019703),

which could improve outcomes. Additionally, transcriptomic analysis suggested a subset of sarcomas with an angiogenic signature[20], thus upcoming correlative analysis from previously conducted trials[4,36] may help to identify a subset of sarcomas benefitting from this approach. Despite this, we still must identify novel strategies to address the majority of sarcomas which lack tumor immunogenicity, the ability of the sarcoma cells to induce and maintain an early immune response.

The main combination strategies aimed at inducing tumor immunogenicity include cytokines, radiation, and cytotoxic chemotherapy together with ICIs. A recent trial combined an IL-2 pathway agonist, NKTR-214, with nivolumab in a large phase I/II study for bone and soft tissue sarcomas (NCT03282344). While early results showed modest responses[75], correlative studies presented in the abstract suggested candidate biomarkers, including changes in PD1/PD-L1 expression in tumor and immune cells with treatment, as well as genetic variations apart from TMB that may impact outcomes[75]. The full publication is anxiously awaited as this large study may further help elucidate mechanisms of response and resistance. Additionally, Zhang *et al.*[76] showed in a phase 0 trial that systemic use of interferon-gamma could increase MHC-I expression and T-cell infiltration in patients with synovial sarcoma and myxoid round cell liposarcomas, leading to a phase II trial of interferon-gamma with pembrolizumab (NCT03063632). While no results are yet published for this study, this is another unique strategy to target early immune responses and restore immunogenicity in cold sarcomas. Another mechanism to boost tumor necrosis and early immune responses is radiation therapy, and the final results of an ongoing study of preoperative radiation therapy for stage III UPS and dLPS with or without concurrent/adjuvant pembrolizumab (NCT02301039) are likely to contribute greatly to our understanding of the immune impact of this critical component of therapy for newly diagnosed sarcomas.

Finally, we will now discuss the emerging strategy of combining checkpoint blockade with cytotoxic chemotherapy, to induce tumor immunogenicity, neoantigen production, and trigger an early immune response, in hopes of potentiating the downstream activity of ICIs.

Chemotherapy combinations

One of the most potential inducers of tumor immunogenicity is traditional chemotherapy, with various agents inducing immunogenic cell stress, production of innate immunity attractants including type 1 interferons, and inflammasome induction[77]. In particular, doxorubicin has been shown in a variety of preclinical tumor models to potently induce the production of DAMPs and type 1 interferons promoting downstream transcription of interferon-stimulated genes, and ultimately increased DC and T cell infiltration in tumor deposits[78-83]. Other commonly used cytotoxic therapies in sarcomas, including gemcitabine, trabectedin, and eribulin, also have pro-immunogenic effects. Gemcitabine suppresses MDSCs within other cancer subtypes[84], trabectedin has been shown to diminish the production of immunosuppressive cytokines in myxoid liposarcomas[85], and eribulin helps to remodel aberrant tumor blood vessels in murine mouse models[86]. In hopes of providing a source of immunogenic neoantigens and pro-inflammatory mediators for poorly immunogenic sarcomas, multiple studies exploring chemotherapy with checkpoint inhibitors have been recently reported or are ongoing.

Two studies have recently been published reporting very promising activity of combination doxorubicin with pembrolizumab. Pollack *et al.*[32] reported a median PFS of 8.1 months in 37 patients with bone and soft tissue sarcomas, with a 73% progression-free rate at 24 weeks, despite an objective response rate of only 19%. Limited correlative data were reported with this study, but suggested that the presence of TILs was associated with inferior PFS. No additional phenotyping was reported to explain this surprising finding. More recently, Livingston *et al.*[87] reported an overall response rate of 36.7%, median PFS of 5.7 months,

and a 6 month PFS rate of 44%, in 30 patients with advanced soft tissue sarcomas. Interestingly, analysis of PD-L1 expression in 29 of the 30 patients revealed an association of high PD-L1 expression with objective response rate, however, did not correlate with PFS or overall survival. The presence of TILs was not associated with any clinical outcomes, however, no phenotyping was reported with this finding. Ongoing clinical trials are exploring additional doxorubicin combinations, including doxorubicin with ifosfamide and sintilimab (anti-PD-1, NCT04356872), liposomal doxorubicin with ifosfamide and camrelizumab (anti-PD-1, NCT04606108), and doxorubicin with balstimilab (anti-PD-1) and zalifrelimab (anti-CTLA4, NCT04028063).

Regarding other cytotoxics, multiple studies are evaluating gemcitabine with checkpoint inhibitors for sarcomas (NCT04577014, NCT03123276), as well as a study combining metronomic gemcitabine with doxorubicin, docetaxel, and nivolumab (NCT04535713). Results of a study combining first-line trabectedin, ipilimumab, and nivolumab have been reported, with a best overall response rate of 22%, and median PFS had not been reached at the time of the presentation (NCT 03138161)[88]. Finally, an ongoing study of eribulin plus pembrolizumab has shown modest responses in leiomyosarcoma[89], with the full study still pending results (NCT03899805).

Overall, the combination of chemotherapy with checkpoint inhibitors appears the most promising strategy to date to reverse innate immune resistance of most adult sarcomas. However, correlative analysis of these studies to further confirm underlying biomarkers of response and resistance is still in infancy, with many unanswered questions.

CONCLUSION

In summary, there is still a tremendous amount to learn about resistance to immune therapy in sarcomas, but early studies have shown conclusively that for a subset of patients, immune therapy, including checkpoint inhibitors or adoptive cellular therapies, can be highly effective. Emerging research in looking at the underlying genetic and immune environments of sarcomas suggests that the pivotal mechanism of resistance is a lack of underlying tumor immunogenicity. Based on our prior experience, it appears unlikely that a single agent in combination with immune agents will be sufficient to reverse these hurdles for all sarcomas, but the promising activity of cytotoxic chemotherapy and potentially radiation combinations may eventually emerge as a crucial part of our therapeutic armamentarium. It remains critical that all early phase clinical trials of immune therapy include correlative studies, to ensure that we are learning the most from every patient, and to pool these data to understand mechanisms of response and resistance, to inform the next generation of clinical trials.

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Authors' contributions

Made substantial contributions to conception and design of the review and authored the manuscript: Florou V

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Potential mechanisms of resistance to current anti-thrombotic strategies in Multiple Myeloma

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Abstract

Multiple Myeloma (MM) is a common haematological malignancy that is associated with a high rate of venous thromboembolism (VTE) with almost 10% of patients suffering thrombosis during their disease course. Recent studies have shown that, despite current thromboprophylaxis strategies, VTE rates in MM remain disappointingly high. The pathophysiology behind this consistently high rate of VTE is likely multifactorial. A number of factors such as anti-thrombin deficiency or raised coagulation Factor VIII levels may confer resistance to heparin in these patients, however, the optimal method of clinically evaluating this is unclear at present, though some groups have attempted its characterisation with thrombin generation testing (TGT). In addition to testing for heparin resistance, TGT in patients with MM has shown markedly varied abnormalities in both endogenous thrombin potential and serum thrombomodulin levels. Apart from these thrombin-mediated processes, other mechanisms potentially contributing to thromboprophylaxis failure include activated protein C resistance, endothelial toxicity secondary to chemotherapy agents, tissue factor abnormalities and the effect of immunoglobulins/"M-proteins" on both the endothelium and on fibrin fibre polymerisation. It thus appears clear that there are a multitude of factors contributing to the prothrombotic milieu seen in MM and further work is necessitated to elucidate which factors may directly affect and inhibit response to anticoagulation and which factors are contributing in a broader fashion to the hypercoagulability phenotype observed in these patients so that effective thromboprophylaxis strategies can be employed.

Keywords: Multiple myeloma, heparin resistance, thromboprophylaxis, venous thromboembolism, thrombin generation testing, endothelium, immunoglobulin

INTRODUCTION

Background

Multiple Myeloma (MM) is a common haematological malignancy that remains a significant therapeutic challenge despite major advances in both treatments and biological understanding of the disease. Almost all patients will suffer relapse and in conjunction with this, MM is associated with a high rate of venous thromboembolism (VTE). It has long been acknowledged that there is a strong association between cancer and thrombosis but the risk of these events varies widely across the spectrum of cancer subgroups. VTE rates remain high in patients with MM with almost 10% of patients suffering from thrombosis during their disease course[1,2].

The occurrence of a thrombotic event in patients with MM can be associated with significant morbidity and can lead to interruption of treatment, requirement for anticoagulation use and indeed may preclude the use of certain agents in future treatment regimens. Certain studies have also suggested that patients with MM who suffer a thrombotic event are also more likely to have poorer overall survival though further confirmatory data is awaited in this area[3,4]. In light of these issues, optimisation of thromboprophylaxis and further understanding of the mechanisms conferring resistance to and failure of current anti-thrombotic strategies is an ongoing critical area of research [Figure 1].

Treatment of MM

The treatment of MM has rapidly evolved over the past number of years with many therapeutic options now available. Triplet/three drug combinations are the initial standard of care for induction therapy though choice of treatment will depend on factors such as patient fitness, local treatment guidelines and availability/funding of therapy[5,6]. Generally, in younger, fitter patients who may be eligible for autologous transplantation (ASCT), initial therapy will comprise of a proteasome inhibitor such as bortezomib and either an immunomodulatory (IMiD) agent such as lenalidomide or an alkylating agent such as cyclophosphamide. These drugs are usually combined with dexamethasone, a corticosteroid, for maximum efficacy[5]. There are also very efficacious treatments available for older patients or those who may not be fit for ASCT including a combination of lenalidomide and dexamethasone or VMP therapy (bortezomib, melphalan and prednisolone)[5,7]. Upon relapse of disease, treatment options include carfilzomib (a second generation PI), alternative IMiDs such as pomalidomide and anti-CD38 monoclonal antibodies including daratumumab and isatuximab[5,7]. It is well recognised that IMiD therapy confers a high thrombotic risk to the patient, particularly when combined with high doses of steroids, but the exact thrombotic risks of many other agents have been less well characterised[8,9].

Risk assessment of venous thromboembolism

Patients with MM have a multitude of risk factors for development of thrombosis. These can generally be categorised into patient related factors such as prior VTE and increased body mass index (BMI), MM related factors (e.g., renal impairment, hyperviscosity, disease activity, reduced mobility) and therapy-related risk factors. There are a number of models and predictive scores that have attempted to stratify individual risk for VTE. The Khorana score is widely used to identify risk for cancer-associated VTE in patients with malignancy who are initiating systemic therapy. It is comprised of five clinical and laboratory indices, including type of cancer, platelet count, haemoglobin level and white cell count (WCC) as well as BMI[10]. However, given that it is largely based on solid organ tumour data, it has been recognised that this score likely has limitations in the MM population with several studies observing that it does not accurately

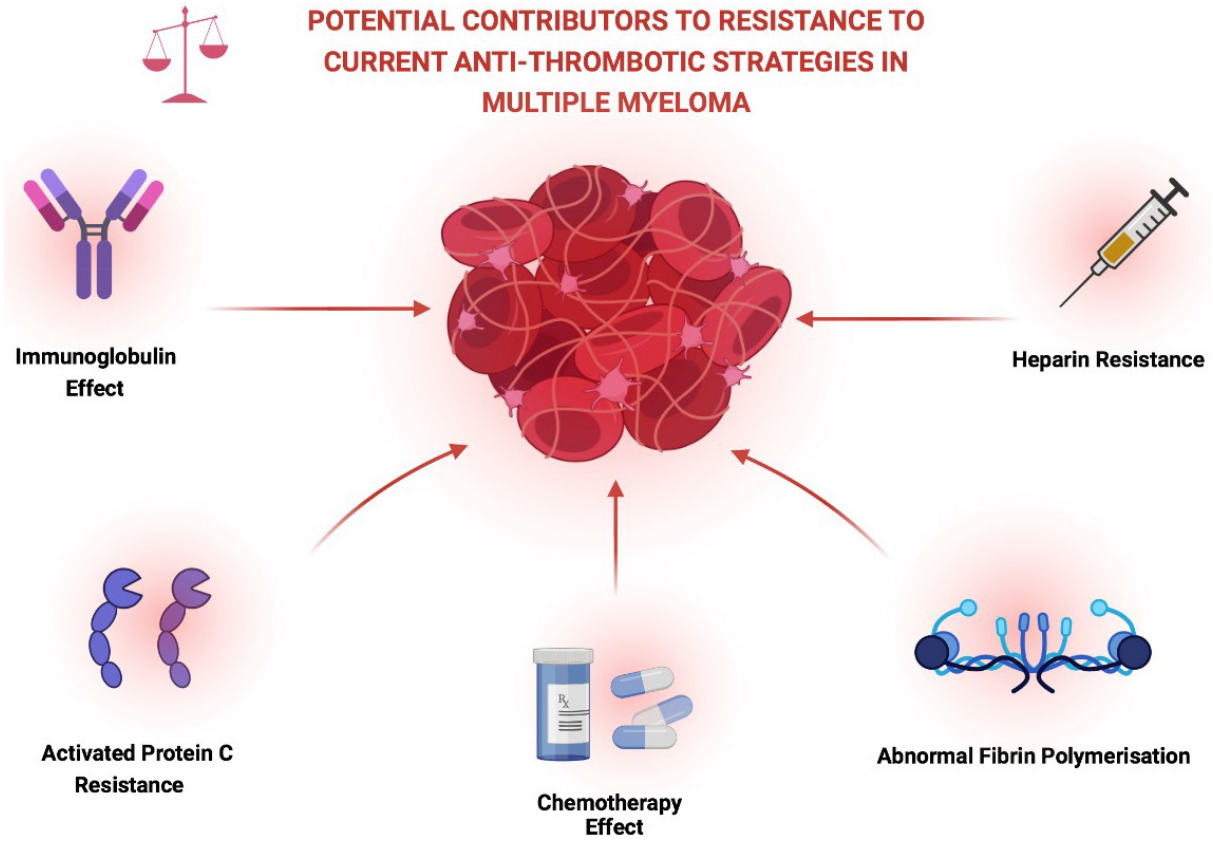

Figure 1. Description of some of the possible contributory factors to the persistently elevated VTE rates seen in multiple myeloma. Created with BioRender.com.

predict VTE risk within this cohort[2,11,12]. In fact, in a study of over 300 patients with MM, Barrett *et al.*[2] noted that WCC was the only single variable from the Khorana score that retained predictive significance for VTE in their cohort.

The current International Myeloma Working Group (IMWG) has provided guidance, based on stratification by Palumbo *et al.*[13], on thromboprophylaxis in MM by compiling a VTE risk assessment incorporating individual, therapy- and myeloma-related factors. This guidance has also been incorporated into the National Comprehensive Cancer Network (NCCN) guidelines but several groups have observed that these guidelines do not fully capture VTE risk and thus have sought to develop novel and more rigorous VTE risk assessment tools [Table 1]. Sanfilippo *et al.*[12] explored individual risk factors for VTE and combined the relevant factors to develop a risk prediction model called the "IMPEDE VTE" score which attributes scores to each contributory risk factor. Similarly, Li *et al.*[14] evaluated the performance of the current NCCN guidelines in a large population based cohort while simultaneously developing their own VTE risk-assessment model specifically for patients undergoing therapy with IMiDs. They concluded that their own model, the SAVED score, which incorporated recent surgery, Asian ethnicity, prior history of VTE, age > 80 and dexamethasone dose had a greater discriminative power than the current consensus recommended by the NCCN guidelines[14]. While these scoring algorithms have advanced VTE-risk discrimination in MM, some concerns have been raised that these scores were designed using retrospective data which was based upon therapies that are now less commonly used[15]. Incorporation of additional biomarkers may further improve the accurate risk prediction of VTE for this vulnerable cohort.

Table 1. Recently developed risk stratification tools for VTE in multiple myeloma

IMPEDE VTE score Variable	Score	SAVED score Variable	Score
IMiD (immunomodulatory agent)	4	Recent **s**urgery (within last 90 days)	2
Body **m**ass index (>/ 25 kg/m^2)	1	Ethnicity: **A**sian race	-3
Fracture (**p**elvic, hip or femur)	4	Prior history of **V**TE	3
Use of **e**rythropoietin-stimulating agent	1	Age "**e**ight" > 80	1
Use of **d**oxorubicin chemotherapy	3	**D**examethasone use (standard or high)	1 or 2
Use of **d**examethasone (high-dose/low-dose)	4/2	**Low risk: </ 1** **High risk: >/ 2**	
Ethnicity = Pacific Islander/Asian	-3		
Prior **V**TE (preceding MM)	5		
Presence of **t**unneled line	2		
Current/**e**xisting use of therapeutic LMWH/warfarin	-4		
Current/**e**xisting use of prophylactic LMWH/aspirin	-3		
Low risk: </ 3 **High risk: 4-7** **Very high risk: >/ 8**			

Adapted from Ref.[12,14]. VTE: Venous thromboembolism; MM: Multiple Myeloma.

Current anti-thrombotic regimens in MM

It is well recognised that due to the unacceptably high thrombosis rates in MM, pharmacological thromboprophylaxis is often necessitated to try mitigate this risk. The IMWG recommend the use of aspirin in lower risk patients and either prophylactic-dose low molecular weight heparin or treatment-dose warfarin in patients at higher risk[13]. In more recent times, focus has switched to evaluating the effectiveness of the direct oral anticoagulant medications (DOACs) including the factor Xa inhibitors (apixaban, rivaroxaban) and factor II inhibitors (dabigatran) in prevention of VTE in MM. While DOACs have been shown in several large trials to be effective and safe in both treating and preventing VTE in patients with malignancy, very few studies have sought to confirm this effect specifically in cohorts of MM patients[16-18]. Despite this, a number of small scale studies have cautiously reported their experiences of the safe and effective use of DOACs in MM.

Storrar *et al.*[19] have described their experience of apixaban thromboprophylaxis in 70 patients with MM, all receiving IMiDs as part of induction therapy. These patients were treated with apixaban 2.5 mg twice daily and there were no reports of VTE during the first six months of therapy with low rates of major bleeding also observed. They concluded that apixaban appeared to be a safe and attractive option for this cohort of patients. Similarly, Cornell *et al.*[20] reported their experiences of apixaban thromboprophylaxis in a more heterogenous group of 50 patients with MM undergoing IMiD-based therapy at various timepoints of the disease course. Again, no instances of VTE were observed in the first six months of observation and, reassuringly there were also no instances of major haemorrhage during this time period. They too concluded that low-dose apixaban was safe and well-tolerated as primary prevention of VTE in patients receiving IMiD therapy. These are, however, small-scale studies and larger, prospective studies will be required to provide conclusive evidence for the long term use of DOACs in MM.

VTE rates despite thromboprophylaxis: immunomodulatory agents

The rate of VTE occurrence remains unacceptably high in MM patients with approximately 10% of patients developing a thrombosis during the course of their disease[21,22]. It has been demonstrated that thrombosis

rates in patients with malignancy are highest in the first year following diagnosis and in particular, in MM, thrombosis rates appear to be highest in the first 3 months following diagnosis[1,13,22,23]. It is hypothesised that these initial high rates are due to both accelerated neoplastic activity, increased tumour burden and, with treatment initiation, there may be overwhelming release of procoagulant factors and cytokines once apoptosis ensues. Given the particularly high risk of VTE associated with IMiDs, adjuvant pharmacological thromboprophylaxis is now widely in use. However, despite this, many groups have reported that VTE rates remain persistently elevated. Leclerc *et al.*[22] reported their observations from a cohort of 213 patients who had received IMiD therapy where thrombotic events were reported in 18.3% of patients with more than half of these events occurring during the first three months of therapy, during which time the vast majority of patients were receiving thromboprophylaxis. VTE occurred more often with anti-platelet use rather than with anticoagulation, an observation that was also seen in the MELISSE study[22,24].

Bradbury *et al.*[25] also recently described the thrombosis rates during the Myeloma IX and Myeloma XI trials. Both trials are phase 3 trials incorporating the upfront use of an IMiD and corticosteroid-containing regimens as induction therapy with the Myeloma IX study containing data from 1936 patients and the Myeloma XI study containing data from 4358 patients. Due to the interim development of the aforementioned IMWG risk assessment guidelines, there were higher rates of thromboprophylaxis (80.5% *vs.* 22.3%) in the later Myeloma XI study but despite this, there was only a modest reduction in thrombosis rates in the latter trial. In the Myeloma IX trial, the 6 months cumulative thrombosis incidence rate was 20.7% in the CVAD (cyclophosphamide, vincristine, adriamycin, dexamethasone) group (non IMiD based) and 15.0% in the CTD (cyclophosphamide, thalidomide, dexamethasone) group (IMiD containing) while in the Myeloma XI trial, 6 months cumulative incidence of VTE was 10.7% in the CRD (cyclophosphamide, lenalidomide, dexamethasone) group and 11.7% in the CTD group, thus showing that despite adherence to current thromboprophylaxis guidelines, VTE rates remain disappointingly high[25].

High VTE rates with other anti-myeloma therapies

Despite IMiDs often being regarded as the culpable prothrombotic agent, consideration has to be given to the thrombotic risk posed by other classes of drugs used to treat MM and worryingly, some of the more novel agents appear to be increasingly linked with thrombosis. Carfilzomib is a second generation proteasome inhibitor with reported VTE rates of 5%-14% in clinical trials and although thromboprophylaxis is recommended for MM patients receiving carfilzomib-containing regimens, the most effective thromboprophylaxis strategy remains to be determined, nor has the mechanism underlying carfilzomib-associated thrombosis been adequately studied[26-29]. The findings of Piedra *et al.*[29] suggest that these patients might benefit from a more enhanced thromboprophylaxis regimen. They recently reported their observations from a retrospective study of VTE incidence across different induction regimens used in newly diagnosed MM (NDMM). They compared rates between KRD (carfilzomib, lenalidomide, dexamethasone) + aspirin, RVD (lenalidomide, bortezomib, dexamethasone) + aspirin and KRD + rivaroxaban and found significantly differing rates of VTE depending on thromboprophylaxis agent used with rates of 16.1%, 4.8% and 4.8% observed in the groups respectively. This suggests that carfilzomib may be more thrombogenic than bortezomib (when combined with lenalidomide), a first generation proteasome inhibitor, though this risk appeared to be somewhat mitigated by using a DOAC rather than an anti-platelet agent[29]. In addition to this, recent advances in cellular therapy have led to the availability of anti-BCMA chimeric antigen receptor T (CAR-T) cell therapy in MM. CAR T-cell therapy is associated with high rates of VTE with Parks *et al.*[30] reporting almost 1 in 10 patients with either non-Hodgkin's lymphoma or MM reportedly developing VTE in the first 60 days post CAR-T cell infusion. There is currently a poor understanding of the pathogenesis of same or indeed on the optimal preventative and therapeutic strategies[30].

POSSIBLE MECHANISMS OF RESISTANCE: THROMBIN-MEDIATED

Heparin resistance

Potential mechanisms contributing to heparin resistance

As discussed above, many patients with MM who are deemed at high risk of VTE are administered thromboprophylaxis with LMWH and it is also commonly utilised at therapeutic doses for treatment of thrombotic episodes in MM. Heparin binds to antithrombin and accelerates the interaction between antithrombin and thrombin or antithrombin and factor Xa, both of which should result in inhibition of prothrombotic activity. Heparin resistance is often defined as the need for higher heparin doses to achieve a targeted level of anticoagulation but it may be multifactorial and its identification is complicated by the lack of consensus on the appropriate target levels in conjunction with the optimal method for measuring heparin effect[31].

One of the important mechanisms responsible for heparin resistance is deficiency of antithrombin (formerly known as antithrombin III). Many disease states such as liver failure, sepsis and treatments, such as asparaginase use in acute leukaemia, are associated with reductions in antithrombin levels but the prevalence of antithrombin deficiency in MM is far less well known and has not been evaluated on a large scale basis[31,32]. It is however, well described that significant proteinuria can contribute to thrombotic potential and that reduced serum albumin levels are associated with increased urinary protein loss, including that of antithrombin[33]. This may be of particular significance both in patients with MM and amyloidosis given the high rates of kidney impairment seen in these conditions. Thus, certain groups have highlighted the need for consideration for testing for antithrombin deficiency in patients with MM in the context of thrombosis and significant proteinuria[21].

Aside from antithrombin deficiency, an additional factor that may contribute to heparin resistance is presence of high levels of coagulation Factor VII (FVIII). Increased doses of heparin have been necessitated for effective thromboprophylaxis in patients who exhibit high levels of FVIII such as patients suffering with acute infective/inflammatory conditions such as Covid-19[31]. Increased FVIII levels in MM have been reported by several groups with particular elevations seen in those undergoing IMiD therapy[34-36]. This raises the question that perhaps higher doses of heparin are necessitated in such patients to achieve adequate protective anticoagulation levels and thus reduce the high rates of VTE seen with current standard dosage.

Evaluating heparin resistance with thrombin generation testing

Thrombin generation assays (TGAs) are global haemostasis assays that seek to assess the entire coagulation process and are thought to be more sensitive than a standard coagulation profile given that the endpoint is conversion of fibrinogen to fibrin. They are *in vitro* assays that utilise tissue factor (TF) and phospholipids to activate the coagulation cascade. The concentration of thrombin is thus measured over a defined time period and a curve is generated to illustrate this production. Various measurements can then be extrapolated from the curve to describe thrombin activity such as lag time (time until thrombin initiation), peak thrombin generation and endogenous thrombin potential (ETP) which reflects the thrombin formation capacity of the plasma[37]. Several groups have postulated that patients with MM may be "resistant" to treatment with heparin and have sought to evaluate this resistance using TGAs.

Chalayer *et al.*[38] describe their observations where they sought to characterise the effect of heparin thromboprophylaxis on thrombin generation in a cohort of patient with MM with a view to exploring potential mechanisms of heparin resistance. They compared a group of patients with MM with intermediate/high VTE risk factors with a group of patients hospitalised with respiratory illness who were deemed to have a low thrombotic risk. They observed similar ETP levels in both of the groups and concluded that there was no *in vitro* evidence of specific resistance to LMWH in this particular cohort of

MM patients[38]. Gracheva *et al.*[39] also assessed a variety of global haemostasis assays (including TGAs, thromboelastography and thrombodynamics) in a cohort of 59 patients, 25 with NDMM and 34 patients undergoing stem cell mobilisation. They compared their results with a cohort of healthy individuals and found that that both thrombodynamics and thromboelastography showed hypercoagulability in both sets of MM patients when compared with the healthy cohort. They then assessed TGAs in the patients undergoing stem cell mobilisation who were receiving heparin therapy and found widely varied results for each individual with no demonstration of hypocoagulability in some patients and more worryingly, a shift towards hypercoagulability in over 20% of patients. The authors concluded that the use of heparin therapy, at its current dose, was ineffective in these patients and that it may either indicate inadequate dosage or heparin resistance in this cohort[39]. Given the stark discrepancy between these studies, further research in larger, more heterogenous groups of MM patients would be necessitated to evaluate if heparin resistance is prevalent and clinically relevant in MM.

Predicting VTE risk with thrombin generation assay testing

Thrombin generation has been studied extensively in malignancy and a large prospective observational study, the Vienna Cancer and Thrombosis Study, assessed the predictive value of thrombin generation for VTE in over 1000 patients with a variety of malignancies. They concluded that patients with an elevated peak thrombin (>/ 611 nM) had an increased risk for development of VTE with a hazard ratio of 2.1[37]. However, the numbers of MM patients included in this study were small thus several groups have sought to establish the predictive value of TGAs specifically in MM.

Leiba *et al.*[40] tested thrombin generation in a cohort of 36 patients with MM at various timepoints over a 2.5-year period with the main objective of determining if thrombin generation has a predictive value for VTE. They observed significantly higher ETPs and peak height values in those who developed thrombotic events compare with those who did not. Interestingly, they also noted a gradual increase in thrombin generation parameters in the time period preceding the thrombotic event. Unlike the findings seen by Gracheva *et al.*[39] they noted that anticoagulation therapy was associated with a significant decrease in ETP and peak height values. As previously discussed, there is an excess incidence of VTE in MM in the first year following diagnosis and the authors observed a moderate in increase in TG parameters concomitant with commencement of MM treatment suggesting that this may partially explain the timing of this excess incidence[40]. Overall, they concluded that thrombin generation testing, both at baseline and during therapy, could serve as a predictive tool for thromboembolic events and perhaps may also have a role in monitoring individual response to heparin[40].

Fotiou *et al.*[41] subsequently carried out a larger prospective study of patients with NDMM and sought to explore the hypercoagulability characteristics in the period of treatment naivety pre-induction therapy. They recruited 144 patients with NDMM and compared their results to a group of healthy individuals. One of their outcomes was measurement of thrombin generation and, in direct contrast to the above findings, they found that, overall, thrombin generation was attenuated in the MM patients compared to the healthy individuals with longer lag-times and lower peak values observed. They also found significantly reduced ETPs in the MM cohort indicating an overall reduced enzymatic activity of thrombin. Interestingly, certain lower ETPs appeared to correlate with a higher risk of VTE and the authors concluded that they had identified clinically relevant biomarkers for VTE risk stratification with their hypothesis being that attenuation of thrombin generation should be interpreted as a reflection of endothelial cell activation i.e., "exhausted thrombin generation"[41]. Legendre *et al.*[42] also showed a potentially hypocoagulable state, with reduced peak thrombin and ETP values in a small group of 14 patients who were both chemotherapy and anticoagulant naïve.

Thus, there appears to be a marked discrepancy in the evaluation of thrombin generation in MM with both hyper- and hypo-coagulable states being observed by different groups, depending on timing of the testing and exposure of the patient to chemotherapy agents. Thrombin generation also increases with age and thus matching against younger, healthy control subjects may confound results[42]. Further evaluation in larger studies with stringent age-matching is necessary to determine both the possible predictive value of thrombin generation for VTE and the clinical utility of such testing in monitoring response and resistance to anticoagulant use (see Table 2 for result of thrombin generation testing).

The potential role of thrombomodulin in pathogenesis of thrombosis

Thrombomodulin is a transmembrane glycoprotein which is located on the luminal surface of endothelial cells. It has roles in both regulation of coagulation and inflammation with its main function being to bind to thrombin. Binding of thrombin to thrombomodulin results in loss of thrombin's procoagulant and profibrogenic properties and acquisition of the ability to activate protein C. It has thus been hypothesised that a reduction in thrombomodulin levels in MM may confer a hypercoagulable profile in patients.

Corso *et al.*[43] sought to serially evaluate the changes in coagulation profiles in patients with RRMM both before and during/after treatment with thalidomide. While many markers were not found to significantly vary, they did observe significant variations in thrombomodulin levels. Thrombomodulin levels appeared to be slightly reduced in most patients at baseline but underwent further reductions during initial treatment with thalidomide therapy with the authors thus concluding that reductions in thrombomodulin levels may have a pathogenic role in thalidomide-related thrombosis. Fotiou *et al.*[41] also noted a reduction in thrombomodulin levels in their cohort of 144 patients with NDMM. Zappasodi *et al.*[44] subsequently sought to confirm the effect of thalidomide on thrombomodulin levels and evaluated the serial behaviour of thrombomodulin in 26 patients with RRMM who commenced thalidomide and dexamethasone therapy. Unlike their initial study where serum thrombomodulin levels fell during the first month of thalidomide therapy, they did not observe any significant modifications of thrombomodulin levels from baseline during the early stages of therapy. They did however note that the cohort of patients had low median basal values of thrombomodulin and thus concluded that this could perhaps suggest a thrombophilic state intrinsic to the MM disease process rather than then anti-myeloma therapy[44]. Again, these observations are limited not only by low patient numbers but also by lack of evaluation in patients being treated with the newer therapeutic agents that are currently in widespread use.

OTHER POSSIBLE MECHANISMS OF RESISTANCE

APC resistance

Many groups have sought to unravel the prothrombotic phenotype in patients with MM with common observations including significant elevations in von Willebrand Factor (VWF) antigen, Factor VIII, D-dimer and fibrinogen levels in patients with active disease. However, the contributory effect of these abnormalities towards VTE occurrence has not been fully disentangled thus far[15,36,45-49]. Interestingly, abnormalities of activated protein C (APC) appear to be a relatively common phenomenon exhibited in patients with MM and, unlike the above, do appear to significantly contribute to VTE occurrence. The normal biological role of APC is to inactivate Factor Va and Factor VIIIa with APC resistance (APCR) leading to non-cleavage of these factors and resultant increased thrombin generation which can potentially lead to a hypercoagulable state. In the Caucasian population, resistance to APC is one of the most common coagulation abnormalities associated with VTE and generally correlates with the presence of the Factor V Leiden R506Q mutation[50]. However, acquired APCR has been described in association with certain malignancies including MM[51,52].

Table 2. Results of thrombin generation evaluation in patients with MM

Author(s)	Multiple Myeloma patient cohort evaluated	Evaluated heparin resistance	Evaluated predictive potential for VTE	Findings
Chalayer *et al.*[38] 2019	Patients on first line therapy with high VTE risk (n = 6)	Yes	No	No evidence for an in vitro LMWH resistance in those with MM compared to patients without MM
Gracheva *et al.*[39] 2015	Patients with primary MM (n = 25) and patients with MM in remission (n = 34) undergoing blood stem cell mobilization	Yes	No	Possible "heparin resistance" with no heparin effect seen in 22% of patients and hypercoagulability seen in certain patients
Leiba *et al.*[40] 2017	Patients with newly diagnosed disease (n = 13) and patients receiving therapy upon relapse (n = 23)	No	Yes	Patients who had a thrombotic event exhibited significantly higher ETP and peak height values than those who did not have a thrombotic event
Fotiou *et al.*[41] 2018	Patients with newly diagnosed disease (n = 144)	No	Yes	Lower ETP values were associated with VTE occurrence
Legendre *et al.*[42] 2017	Patients with newly diagnosed disease (before treatment, including anti-coagulation) (n = 14)	No	Yes	Hypocoagulable profiles including decreased ETP values

VTE: Venous thromboembolism; MM: Multiple Myeloma; ETP: endogenous thrombin potential.

Given this association, Elice *et al.*[53] sought to fully characterise the incidence of acquired APCR and its correlation with VTE in a large cohort of patients with MM (n = 1178). They recorded APCR in 109 patients (9%) but interestingly, this did not necessarily translate into the presence of factor V Leiden mutations with over half of these patients testing negatively for this mutation. Those with an abnormal APCR ratio but negative DNA testing for the factor V Leiden mutation were considered to have acquired APCR. Within this cohort of patients with acquired APCR, a higher incidence of VTE was observed when compared with controls (31% *vs.* 12%) and furthermore, they also exhibited a lower thrombosis-free survival. The authors thus concluded that the presence of acquired APCR was statistically associated with an increased VTE risk[51,53].

Following on from this, other groups have sought to further unravel the mechanisms behind APCR and isolate the exact causative single nucleotide polymorphism (SNPs) in the endothelial protein C receptor (EPCR). EPCR has a key regulatory role in protein C activity via binding and facilitating the interaction with the thrombin-thrombomodulin complex. Several polymorphisms have been reported in EPCR with the 4678G/C SNP thought to be associated with high levels of circulating APC and reduced risk of thrombosis. Dri *et al.*[54] sought to evaluate the presence of this polymorphism in a cohort of patients with MM who had developed thrombosis and found a significantly lower frequency of the 4678C allele of the EPCR gene in MM patients compared with the known frequency in a healthy adult population. The authors concluded that the reduced frequency of this protective allele could be contributing to overall disease hypercoagulability and that it may be useful to test for same to risk stratify MM patients. However, this was a small study and thus further evaluation of larger series of patients would be necessitated to determine that there is in fact an increased prevalence of this SNP in the MM population and if it indeed has a contributory effect to overall rates of VTE[54].

Endothelial dysfunction and toxicity related to the effect of chemotherapy

With the widespread introduction of several novel therapies for MM, there has been a focus on emergent cardiovascular complications in conjunction with the persistence of high rates of VTE in the MM population[26,55]. Many of the hypotheses surrounding this VTE risk centre on the toxicity that may be caused to the endothelium by certain anti-myeloma agents. Dexamethasone is a corticosteroid that is used in many of the treatment regimens for MM. Studies have shown that combining dexamethasone with other agents increases a patients risk of VTE[8,9]. The reasons behind this are not well understood but there is some *in vitro* evidence that dexamethasone can stimulate the endothelium to increase expression of both VWF and TF[56]. Similarly, IMiD agents and carfilzomib are both thought to activate and cause direct toxicity to the endothelium with a downstream pro-thrombotic effect occurring following release of coagulation factors and cytokines[57,58].

A small number of studies have been carried out specifically evaluating the toxic effects of anti-myeloma chemotherapy agents on endothelial cells (ECs). Early *in vitro* studies of IMiD agents noted that in a previously compromised/injured endothelium, as which would be seen with prior chemotherapy use, thalidomide could alter the expression of thrombin receptor PAR-1 and thus induce endothelial dysfunction and potentially a hypercoagulable state[59]. In a more recent study, Sanchez *et al.*[60] have reported their observations on exposure of ECs to some of the newer MM induction chemotherapy agents and they have shown that these agents can in fact lead to increased VWF and ICAM-1 expression and also an increase in cell permeability, exhibited by reduced VE-cadherin expression and cell monolayer integrity. Interestingly, this effect appeared to be somewhat counteracted by addition of the endothelial protectant agent defibrotide[60], thus raising the hypothesis that perhaps endothelial protection agents could have some anti-thrombotic utility in MM.

The direct effects of M-protein/immunoglobulin on the prothrombotic environment and fibrin clot formation

MM is characterised by high levels of circulating clonal immunoglobulins or "M-proteins" and these immunoglobulins are hypothesised to have the ability to contribute directly to thrombotic risk, not only by direct vascular wall injury but also by interference with fibrinolysis[61]. Fibrinolysis is the breakdown of fibrin within blood clots and is a highly regulated enzymatic process that, if functioning correctly, prevents unnecessary accumulation of intravascular fibrin. Clot stability is dependent upon many factors including local factors such as calcium concentration, thrombin concentration, pH and platelets numbers[62]. It is also influenced by the geometric compilation of the fibrin network and the composition and diameter of the fibrin fibres from which it is constructed[62]. Abnormal fibrin clot structure can lead to prolonged resolution/retraction of a clot[63].

Carr *et al.*[64] originally sought to evaluate the influence of high levels of immunoglobulin on fibrin polymerisation and clot structure *in vitro*, noting that in congenital dysfibrinogenemia, recurrent thrombosis were associated with the presence of abnormally thin fibres of fibrin. They hypothesised that, given that fibrin structures are sensitive to the environment in which they are formed, that high levels of immunoglobulin would interfere with fibrin monomer polymerisation. They characterised the clot formation in MM and , as seen in congenital dysfibrinogenemia, they noted that the clots were associated with the presence of abnormally thin fibrin fibres[64]. They subsequently sought to evaluate the resistance of such clots to fibrinolysis and observed that these clots dissolved at a slower rate and they thus concluded that they were associated with inhibition of fibrinolysis[65].

Undas *et al.*[66] subsequently sought to evaluate fibrin clot properties and their determinants *ex vivo* in a cohort of 106 MM patients. Following collection of peripheral blood from these patients, they performed fibrin clot analysis, measuring variables such as clot permeation, clot compaction, turbidity measurements and also the efficiency of fibrinolysis using plasma clot lysis assays. They observed that plasmin clot variables differed significantly between the MM patients and a cohort of healthy control subjects with changes including higher fibrin fibre densities and reduced tPA-mediated lysability. They hypothesised that higher thrombin generation potentials were likely a major mechanism in these alterations in clot properties and that this abnormal fibrin clot structure likely exhibited a prothrombotic phenotype[66].

The concept that local thrombin concentrations can impact upon the structure of clots with higher thrombin concentrations generating more stable clots which are more resistant to fibrinolysis and may promote thrombosis has been well described[62,63,67,68]. It thus appears possible that in MM, given the duel pathology of both high circulating immunoglobulin levels and potentially high thrombin generation, clots produced could be less susceptible to fibrinolysis and thus perhaps more resistant to the current anticoagulant strategies that are employed.

Tissue factor

TF is an essential glycoprotein that serves as principal initiator of coagulation. TF-mediated conversion of Factor IX to its activated form is pivotal for effective haemostasis while disruption of the endothelium causes exposure of TF-expressing endothelial cells and thus facilitates binding of factor VII[69]. Apart from its roles in coagulation, TF has also been implicated in both tumour spread and angiogenesis and in conjunction with this, high levels of TF have also been described in several solid organ malignancies with conflicting reports on its potential utility as a biomarker for VTE occurrence[70]. TF can also be demonstrated on circulating microparticles (MPs) which are small, procoagulant membrane vesicles that are released from cells during activation or during apoptosis. These MPs are found in low steady state concentrations in healthy individuals and increase during times of inflammation. MPs carrying TF express platelet selectin glycoprotein ligand-1 which binds p-selectin on the surface of activated endothelial cells[70].

In light of the above, several groups have sought to establish the role of TF in MM disease. Papageorgiou *et al.*[71] investigated the role of TF and MP-TF *in vivo* in human myeloma cell lines. They determined that the expression of the TF gene, the presence of the TF protein on cell membrane and the procoagulant activity of TF were all detectable. They also observed that these cells could release MPs into their microenvironment and that these MPs conferred markedly increased procoagulant activity, expressing twofold higher levels of TF as compared to the original cells. The authors thus concluded that the hypercoagulability observed in MM may be enhanced by myeloma cell derived MPs released into the microenvironment[71].

Other groups sought to evaluate the TF activity in patients with MM with Auwerda *et al.*[72] evaluating this in a cohort of 122 patients with MM. They found that MP-TF activity levels were increased in the MM cohort in comparison to healthy volunteers. They performed serial evaluation of these activity levels and found that MP-TF activity levels showed a reduction post-induction therapy in many of the patients, but interestingly, levels remained elevated in those who had suffered a thrombotic episode[72]. Nielsen *et al.*[73] also sought to explore the role of TF in MM by isolating extracellular vesicles (EVs) from the peripheral blood of 20 patients with MM and subsequently demonstrating substantially higher thrombin generation and TF activity when compared with healthy control subjects. Similar to the above, they also performed serial activity levels and noted that the procoagulant activity of the EVs diminished after treatment, though of note, neither of the treatment groups that they evaluated contained the more thrombogenic IMiD agents

(treatment groups = bortezomib, cyclophosphamide and dexamethasone and melphalan, prednisolone and bortezomib)[73].

All groups concluded that their findings may, at least in part, explain why there is an increased risk of VTE in MM but the prothrombotic contribution of TF in MM would need to be confirmed in larger cohorts of patients. In addition to this, TF activity is not routinely measured in clinical practice and a standardised assay would have to be developed and validated before it could be considered for widespread use as a biomarker.

CONCLUSION

It is clear that rates of thrombosis remain high in MM and, unfortunately, current thromboprophylaxis regimens do not entirely abrogate this risk of VTE. Thus the gap in knowledge between the persistently high levels of VTE and the inadequacies in current preventative measures needs to be bridged.

It is also increasingly evident that there are a multitude of factors contributing to the prothrombotic milieu seen in MM, many of which cannot be evaluated in a clinical setting. Further work is necessitated to elucidate specific pathways and factors contributing to the hypercoagulable phenotype observed in these patients. Additional clinical studies should focus on identifying novel biomarkers of VTE risk in MM and developing effective screening methods for anticoagulant resistance. Taken together, these approaches may help inform therapeutic strategies to overcome these mechanisms of resistance and provide effective thromboprophylaxis for patients with MM.

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Cancer Drug Resistance

Commentary

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Combating CHK1 resistance in triple negative breast cancer: EGFR inhibition as potential combinational therapy

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Abstract

Triple negative breast cancer (TNBC) is marked by a lack of expression of the Estrogen Receptor, Progesterone Receptor, and human epidermal growth factor receptor 2. Therefore, targeted therapies are being investigated based on the expression profiles of tumors. Due to the potential for acquired and intrinsic resistance, there is a need for combination therapy to overcome resistance. In the article by Lee et al., the authors identify that, while prexasertib (a CHK1 inhibitor) lacks efficacy alone, combination with an EGFR inhibitor provides synergistic anti-tumor effects. Advances in targeted therapy for TNBC will benefit the clinical landscape for this disease, with this study initiating a new avenue of investigation.

Keywords: CHK1, TNBC, EGFR, chemoresistance

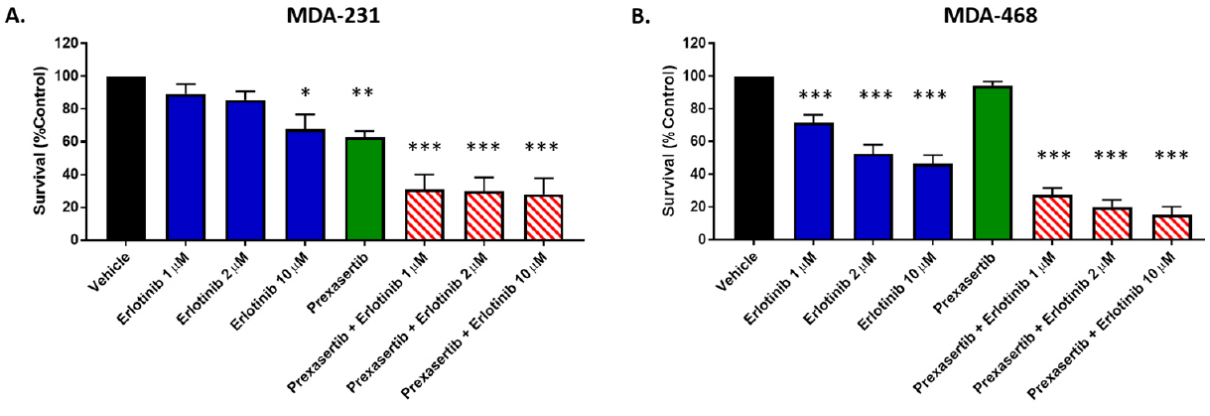
In the recent article by Lee *et al.*[1], the primary goal of the authors was to identify a novel targeted therapy for triple negative breast cancer (TNBC). They are focused on the interaction and cooperation between epidermal growth factor receptor (EGFR) and CHK1. Prior to this study, it was well established that CHK1 inhibition (using prexasertib) increased cell death by blocking the cell cycle checkpoints. In addition,

previous studies showed that signaling through the PI3K and MAPK pathways are involved in resistance to prexasertib in sarcomas. Furthermore, prexasertib has been used in combination with PI3K inhibitors to cause changes in survival and apoptosis in TNBC[2]. The authors mention that while prexasertib is effective as a combination therapy, its use as monotherapy has been less successful in part due to drug resistance. It's unclear, however, whether the resistance is related to CHK1 expression, as the CHK1 expression in Supplementary Figure 1 does not appear to correlate to the efficacy of prexasertib.

In this paper, the authors test a panel of TNBC cell lines, and show that the MDA-MB-468 cells are highly resistant to prexasertib, whereas the MX-1 cells are sensitive[1]. Two cell lines used in this panel, MDA-MB-468 and HCC1937, are both Rb-deficient. This deficiency could impact CHK1, as a 2018 paper demonstrated that Rb-deficient cells express higher levels of CHK1 and are more sensitive to CHK1 inhibitors[3]. They chose to move forward with the MDA-MB-468 (resistant) and MDA-MB-231, which had moderate prexasertib sensitivity, for the remainder of their studies. They investigated the MAPK/PI3K pathways and found EGFR elevated in the resistant MDA-MB-468 cells, while AKT was elevated in both the MDA-MB-468 and the sensitive MX-1 cells. They chose to further investigate the function of EGFR in mediating prexasertib resistance, based on the expression in resistant cells. Using the MDA-MB-231 cells, they show that treatment with EGF increased resistance to prexasertib; however, it would be interesting to know whether this treatment altered receptor level or just downstream signaling. In addition, there is no dose dependence of EGF on prexasertib response, suggesting that they have reached a threshold of EGF/EGFR stimulation. As an aside, this is likely reached in the MDA-MB-468 cells as well, given that the higher dose of EGF increased sensitivity to prexasertib.

To overcome the resistance, they used a combination treatment of prexasertib with an EGFR inhibitor, erlotinib. This combination regimen showed synergy in both the resistant MDA-MB-468 and the moderately resistant MDA-MB-231 cells. Mechanistically, the authors state that there are changes in phosphorylation of Bad; however, the blots appear to show changes in the total protein. In addition to Bad, the MDA-MB-468 cells also show a decrease in phosphorylation of AKT, which is not observed in the MDA-MB-231 cells (likely due to their low baseline levels). *In vivo* studies showed that the combination of both drugs was required to decrease tumor weight in the mice injected with MDA-MB-468 cells, as opposed to single-drug treatments alone[1]. While these results are contradictory to the *in vitro* results from Figure 1 (reprinted from Figure 4 of Ref.[1]), which show that erlotinib alone could decrease cell survival in the MDA-MB-468 cells, this could be due to cell non-autonomous interactions that would be lacking *in vitro*.

The overall message from the manuscript is that EGFR inhibition can be used to overcome resistance to CHK1 inhibition. The manuscript by Lee *et al.*[1] gives great promise and lays the foundation for further investigation into this important mechanism of treating TNBC. To aid in the understanding of the relationship between EGFR and CHK1 in TNBC, an investigation of the sensitive MX-1 cells with EGF treatment would be beneficial. A deep dive into the correlation of EGFR and CHK1 in human TNBC would also be interesting to determine potential resistance to CHK1 inhibitors. In addition, previous investigations have shown the benefit of ATR and/or PARP inhibitors with prexasertib[4] (also discussed in clinical trial NCT04032080), which may provide additional investigative avenues. Regardless, the clinical need for better targeted therapies for TNBC is high, and Lee *et al.*[1] have identified a novel combination regimen using EGFR inhibition with CHK1 inhibition.



C.

MDA-231			MDA-468		
Prexasertib (nM)	Erlotinib (μM)	CI Value	Prexasertib (nM)	Erlotinib (μM)	CI Value
10	1	0.37	10	1	0.19
20	1	0.52	20	1	0.13
10	2	0.38	10	2	0.15
20	2	0.51	20	2	0.11
10	10	0.50	10	10	0.27
20	10	0.53	20	10	0.16

Figure 1. Erlotinib co-exposure with prexasertib synergistically enhanced cell killing. Viability of 3D cultures of MDA-231 (A) and MDA-468 (B) after exposure to prexasertib, erlotinib, and combinations of erlotinib and prexasertib; the combination index (CI) showed synergistic interaction (CI < 1) of erlotinib and prexasertib in both MDA-231 and MDA-468 (C).

DECLARATIONS

Authors' contributions

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

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Consent for publication

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Cancer Drug Resistance

Review

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Lysosome-mediated chemoresistance in acute myeloid leukemia

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Abstract

Despite the outstanding advances in understanding the biology underlying the pathophysiology of acute myeloid leukemia (AML) and the promising preclinical data published lastly, AML treatment still relies on a classic chemotherapy regimen largely unchanged for the past five decades. Recently, new drugs have been approved for AML, but the real clinical benefit is still under evaluation. Nevertheless, primary refractory and relapse AML continue to represent the main clinical challenge, as the majority of AML patients will succumb to the disease despite achieving a complete remission during the induction phase. As such, treatments for chemoresistant AML represent an unmet need in this disease. Although great efforts have been made to decipher the biological basis for leukemogenesis, the mechanism by which AML cells become resistant to chemotherapy is largely unknown. The identification of the signaling pathways involved in resistance may lead to new combinatory therapies or new therapeutic approaches suitable for this subset of patients. Several mechanisms of chemoresistance have been identified, including drug transporters, key secondary messengers, and metabolic regulators. However, no therapeutic approach targeting chemoresistance has succeeded in clinical trials, especially due to broad secondary effects in healthy cells. Recent research has highlighted the importance of lysosomes in this phenomenon. Lysosomes' key role in resistance to chemotherapy includes the potential to sequester drugs, central metabolic signaling role, and gene expression regulation. These results provide further evidence to support the development

of new therapeutic approaches that target lysosomes in AML.

Keywords: Lysosome, chemoresistance, AML, lysosomotropic drug, lysosomal sequestration, refractory AML

INTRODUCTION

Relapse and refractory diseases are major clinical challenges during the management of acute myeloid leukemia (AML) patients, and they prevent an optimized response to current treatments. As relapse refers to reappearance of the disease, relapse episodes are strongly related to refractoriness, both of them leading to poor prognosis[1]. For the last five decades, the standard AML therapy consisted of a combination of cytarabine and an anthracycline[2], and improvements in the survival rate are mainly due to optimization of the supportive care and hematopoietic cell transplantation protocols. Recently, new targeted drugs have been approved, incorporating the notion of personalised treatments for AML. Continuous assessment of newly approved drugs over time will provide valuable complete efficacy and safety data that will result in an optimal drug regime, as current clinical benefit is controversial[3,4]. Thus, acquisition of new biological insight in AML pathophysiology represents an unmet need necessary to expand the targetable therapeutic mechanism repertoire to overcome chemoresistance and, then, significantly improve clinical outcomes for AML patients.

Primary and secondary chemoresistance have been widely explored using conventional approaches, searching for gene mutations, chromosomal aberrations, and dysregulated signaling pathways[5-8]. Changes in the multidrug resistance gene family affect the intracellular concentration of drugs by either reducing the active transport into the tumor cells or increasing the efflux out to the extracellular space. Other mechanisms of action that affect the response to chemotherapy include: modifications in the chemotherapy molecular targets, preventing the pharmacologic action, increased ability to repair tumor DNA damage, defective response to proapoptotic stimuli, and changes in the tumor microenvironment[8]. Although several inhibitors targeting drug-resistance mechanisms have been reported, their clinical development is still under evaluation.

Oxidative phosphorylation function, metabolic plasticity, and mitochondrial adaptation contribute to chemoresistance in AML, especially towards cytarabine, as resistant AML cells rely more on mitochondrial oxidative phosphorylation and less on glycolysis[9,10]. Increased mitochondrial mass, mitochondrial membrane potential, reactive oxygen species production, and a characteristic gene signature associated with oxidative phosphorylation are hallmarks of chemoresistance AML cells[9,11]. Indeed, inhibition of oxidative phosphorylation induces chemosensitivity[9,11-13]; and mitochondrial oxidative phosphorylation and respiratory capacity correlate with a better response to cytarabine treatment in AML cells[11]. This metabolic reprogramming might have an important therapeutic implication and metabolic vulnerabilities might be exploited pharmacologically.

Closely related to mitochondria, lysosomes have attracted special interest in oncology due to their growing importance in transformation processes. The traditional view of lysosomes has been challenged by the recognition that lysosomes are not only "degradative organelles", but also metabolic sensors and regulators, becoming legitimated as intracellular signaling hubs[14]. Additionally, recent findings highlight the physical and functional interaction of mitochondria and lysosomes, suggesting that this crosstalk plays a major role in metabolic regulation, based on the transfer of Ca^{2+} between organelles[15,16], affecting the cellular response to treatment. In this review, the role of lysosomes in chemoresistance in AML is discussed and an overview of the potential therapeutic approaches for overcoming refractoriness in leukemia is provided.

LYSOSOMES

Lysosomes were first described in the 1950s as organelles responsible for the degradation of biological macromolecules from extra- and intra-cellular origins[17]. Their central role in cellular recycling and homeostasis was revealed later when autophagy was discovered[18,19]. Recent discoveries confirm lysosomes as crucial modulators of cell homeostasis, regulating both cellular metabolism and clearance (reviewed in Ref.[20]).

Structurally, lysosomes are acidic organelles surrounded by a phospholipid bilayer. The acidic lumen is maintained by vacuolar-type H+ ATPase (V-ATPase) on the lysosomal membrane[21]. Other key proteins on lysosomal membrane are: lysosome-associated membrane protein, soluble N-ethylmaleimide-sensitive factor activating protein receptors, toll-like receptors, and mammalian target of rapamycin (mTOR)[22]. Luminal hydrolytic enzymes of lysosomes include proteases, sulfatases, nucleases, lipases, phosphatases, and nucleases, which degrade macromolecules.

Lysosomes belong to the endolysosomal system, a dynamic network of organelles consisting of early, late, and recycling endosomes and lysosomes. Primary lysosomes originate from the Golgi apparatus. Early endosomes formed from the plasma membrane might also progress to late endosomes and lysosomes, as a result of a maturation process. Alternatively, a contact site between lysosomes and late endosomes can be formed, followed by cargo transfer and dissociation (kiss-and-run model), or late endosomes can fuse with lysosomes, creating a hybrid organelle that subsequently evolves in lysosomes (fusion and fission process)[23].

During malignant transformation, cancer cells adapt their physiological processes to sustain their intrinsic anabolic and catabolic needs. Both lysosomal mass and subcellular localization are widely changed to enable the acquisition of cancer cells' idiosyncratic feature of uncontrolled growth. Recycling of exogenous material provides energy and key molecular components, while autophagy enhances catabolism and, consequently, energy and metabolite precursors are supplied. Nutrient sensing is tightly regulated by lysosomes, based on the activation and translocation of the mTORC1 complex to the lysosome membrane, enhancing lipid catabolism under starving condition in transformed cells (reviewed in Ref.[24]).

Lysosomes in AML

During leukemogenesis, AML cells increase their lysosomal mass, although their number is not significantly affected[25]. As AML relies on fatty acids for energy supply, lysosome-dependent fatty acid oxidation rate is higher, inducing an augmented lysosomal mass to support this process[26]. The gene network that regulates the lysosomal biogenesis is also upregulated, similarly to the expression of key lysosomal enzymes[27-37]. Indeed, the lysosomal matrix enzyme activity is enhanced in AML, as compared to healthy myeloid cells, probably due to an increase in the quantity of enzymes and the influx rate[38]. As a consequence of these lysosomal changes, AML cells contain fragile lysosomes due to destabilization of the lysosomal limiting membrane and lower pH.

V-ATPases play both direct and indirect roles in the control of cellular signaling. Growth, survival, and differentiation signaling pathways frequently rely on these ATP-dependent proton pumps. Control of vesicular pH by V-ATPase is essential for proper signaling by many plasma membrane receptors that traffic through the recycling networks, including Notch and Wnt[39]. Canonical Wnt is required for the development and maintenance of AML[40-42]. Inhibition of V-ATPase prevents the activating phosphorylation of the Wnt receptor upon ligand binding and dysregulates ligand-mediated internalization of the receptor[43,44]. Although the importance of Notch as a therapeutic target in AML is still controversial,

this signaling pathway regulates proliferation and cell survival[45-47]. Activation of the Notch receptor induces the intracellular domain to be cleaved and translocated to the nucleus, a process requiring V-ATPase function[48-50]. Similarly, PI3K/Akt/mTOR signaling pathway is crucial to many physiological processes, such as proliferation, gene expression regulation, differentiation, cell death, metabolism, cell survival, and migration, and it is frequently hyperactivated in AML[51-55]. While the precise mechanism involved in the V-ATPase-mediated modulation of mTOR remains widely unknown, inhibition of V-ATPase represses mTOR activation[56], and mTOR inhibition leads to AML cell death[57,58].

Lysosomal sequestration of drugs

Lysosomal sequestration or lysosomal trapping is an important mechanism responsible for chemoresistance acquisition[59]. Many chemotherapeutics used in clinics (i.e., anthracyclines, taxanes, platinum-based drugs) are lipophilic, weak-base drugs and can therefore diffuse freely across lipid membranes, including the plasma membrane and lysosomal membrane. Alternatively, lysosomotropic drugs can be actively transported by inward turned multidrug efflux transporters of the ATP-binding cassette superfamily, embedded in the lysosomal membrane (originally expressed in the plasma membrane and endocytosed into lysosomes), particularly ABCB1[60-63] and ABCA3[38]. The acidity of the lysosomal lumen facilitates the rapid protonation of weak-base drugs, impairing their ability to cross back across lipid bilayers, resulting in their marked lysosomal accumulation and compartmentalization[64,65]. Chemotherapeutics sequestered in lysosomes and associated to drug resistance phenomena include tyrosine kinase inhibitors[66-68], topoisomerase inhibitors[69-71], antimetabolites[72], alkylating agents[73,74], and microtubule-targeting agents[75,76]. Lysosomal sequestration severely affects drug subcellular distribution, significantly reducing efficiency, since lysosomes are seldom the target sites for these chemotherapeutics, and the sequestered drugs will not reach their targets[77]. Therefore, higher concentrations are required to achieve therapeutically relevant concentrations, increasing side effects in patients, and promoting secondary chemotherapy resistance. Treatment with these types of drugs induces expansion of the lysosomal compartment, thereby enhancing their lysosomal sequestration capacity and further increasing chemoresistance, constituting a feedback loop[78-80].

The transcription factor EB (TFEB) is the master regulator of lysosomal biogenesis, by modulating the expression of genes bearing the coordinated lysosomal expression and regulation motif. In resting conditions, phosphorylated TFEB is retained inactivated in the cytoplasm by the 14-3-3 protein. Calcineurin dephosphorylates and activates TFEB, leading to its dissociation from 14-3-3 and subsequent translocation to the nucleus. mTOR phosphorylates (and inactivated) TFEB, enabling its binding to 14-3-3 in the cytoplasm. The activity of calcineurin is modulated by the release of Ca^{2+} from the lysosomes through the lysosomal Ca^{2+} transporter mucopilin (MCOLN1)[81]. mTOR can be inhibited by raising the pH, as lysosomotropic drugs do[82]. Activation of TFEB induces lysosomal biogenesis which increases lysosomal sequestration capacity and exerts a feedback loop.

Clearance of chemotherapeutics sequestered in lysosomes might also provide an additional chemoresistance mechanism. Exocytosis has been proposed as the preferred process[83], as drug accumulation induces an increase of pH, leading to an activation of exocytosis[84,85]. Moreover, drug sequestration-induced TFEB activation partially results in the induction of lysosomal exocytosis and clearance of lysosomal content outside the cell[86,87]. However, once drugs have been released to the extracellular space, they can rediffuse back into the cells, making the exocytosis-mediated chemoresistance a controversial process that requires further clarification.

The anthracyclin daunorubicin (DNR), a backbone chemotherapeutic agent in first-line treatment of AML, displays physicochemical features compatible with lysosomal trapping. Early studies demonstrated that DNR intracellular distribution depends on drug treatment response. Sensitive AML cells preferentially accumulate DNR in the nucleus, where the pharmacology effect is exerted. In contrast, DNR-resistant AML cells tend to sequester DNR in lysosomes[88]. Indeed, expanded lysosomes are observed in response to DNR treatment, as well as a diminished nuclear drug uptake, exhibiting a 2.5/3-fold less DNR concentration in nucleus in resistant versus sensitive AML cells[69]. In AML, the relevance of ABCB1 in the active transportation of DNR into lysosomes is limited[60,69], in contrast to other solid tumor cells[62,63,89]. Instead, in AML, DNR is actively influxed by lysosomal ABCA3, a transporter upregulated in chemoresistant patients and correlated with poor prognosis[37,38].

LYSOSOME-BASED THERAPEUTIC APPROACHES

The key contribution of lysosomes to chemoresistance raises increasing interest in lysosome-targeting therapies to either sensitize tumor cells to current approved chemotherapy or as new pharmacologic approaches. The transformation process itself affects the integrity and size of lysosomes, increasing their fragility. Sphingolipid metabolism alterations are also often found in cancer cells, leading to changes in the lysosomal membrane function and structure[90]. Due to an increased metabolic demand, cancer cells upregulate their lysosomal function, resulting in an augmented lysosomal mass[91]. Accumulation of lysosomotropic drugs in cancer cells destabilizes lysosomes, causing their failure and eventually activating cell death program. However, healthy lysosomes are fully functional and display compensatory mechanisms that prevent fatal damage. Consequently, a wide therapeutic window is found due to these differences in fragility of lysosomes in cancer cells *vs.* healthy cells. Several strategies have been explored, including lysosomal destabilization. Lysosomotropic compounds can accumulate in lysosomes, causing lysosomal membrane-cell permeabilization, release of cathepsins, and consequently, activation of the cell death program[92]. Using different screening approaches, the anti-malaria drug mefloquine[25], cationic amphiphilic antihistamines[93], and σ2 receptor agonist siramesine[94] were described as lysosomal disruptors in AML cells and sensitizers to approved chemotherapeutics. Mefloquine disturbs the lysosomal membrane of AML cells, allowing the release of cathepsins to the cytoplasm and inducing apoptosis[25]. Cationic amphiphilic antihistamines simultaneously disrupt both lysosomes and mitochondria, based on their physico-chemical properties, inducing both apoptosis and autophagy[93]. Both the specific cationic amphiphilic antihistamines and mefloquine spare healthy blood cells, confirming the differential effect of lysosomal disruptors in AML and the existence of a preclinically-validated therapeutic window. However, reprofiling of these drugs to AML is difficult due to their pharmacological profile, and no clinical trials have been successfully completed. Medicinal chemistry programs are expected to be necessary to achieve clinically suitable new compounds. Nevertheless, to date, targeting lysosomal integrity is the most promising therapeutic approach to overcome lysosome-mediated chemoresistance in AML [Figure 1].

Accumulation of chemotherapeutics in lysosomes heavily depends on lysosomal lumen pH. Moreover, in resistant AML cells, the pH gradient between the lysosome and cytosol is higher[95]. Treatment with V-ATPase inhibitor archazolid A induces cell death in leukemic cells, both T-cell acute leukemia and acute myeloid leukemia[96]. Similar results were obtained with bafilomycin A, another V-ATPase inhibitor, although the mechanism of action responsible for this pharmacological effect is still controversial[97], as bafilomycin A is unable to resensitize cytarabine-resistant cells[98]. However, the preclinical data suggest that the therapeutic window was narrow, and their clinical significance might be limited.

As for further lysosome regulators, rapamycin and other mTORC1 modulators, have shown promising results in preclinical assays, specially in combination therapies[52]. However, mTORC1 regulates key

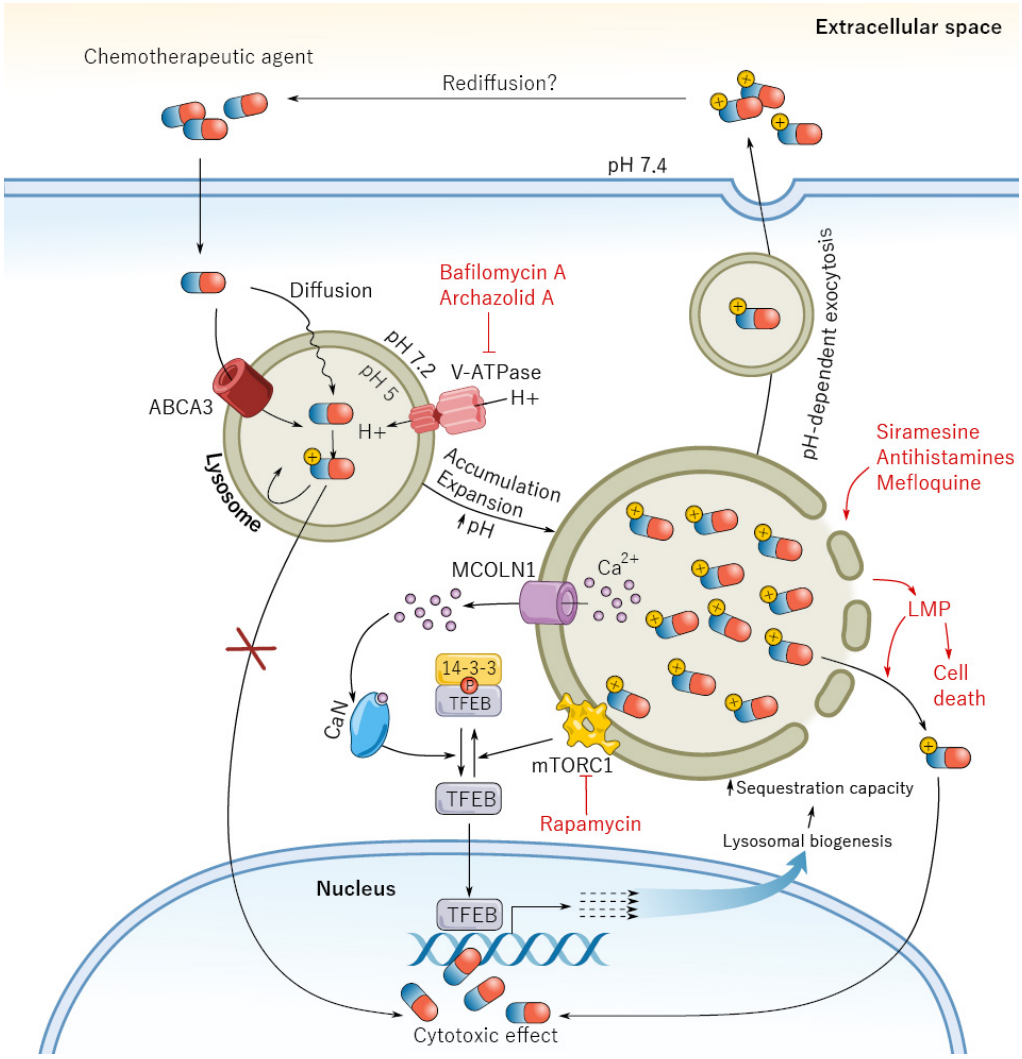

Figure 1. Mechanisms of lysosomal-mediated chemoresistance in acute myeloid leukemia (AML) at a glance. Most chemotherapeutic agents get readily sequestered in lysosomes upon entry in AML cells, causing a remarkable expansion of the lysosomal compartment. Lysosomal expansion is accompanied by an increase in pH, inducing exocytosis and, consequently, clearance of chemotherapy from cells. Both mechanisms prevent chemotherapeutic agents from directly interacting with their molecular targets, commonly located in the nucleus. To revert the undesirable sequestration, two main strategies have been proposed, namely, increasing lysosomal pH by inhibiting V-ATPase or pharmacologically inducing lysosomal membrane leakiness, thus releasing chemotherapeutics and additionally eliciting lysosomal-dependent cell death. Conversely, mTORC1 inhibition contributes to lysosomal biogenesis and sequestration capacity, a mechanism that has been traditionally overlooked in translation of mTORC1 inhibitors and that could partly explain their clinical failure. ABCA3: ATP binding cassette subfamily A member 3; CaN: calcineurin; LMP: lysosomal membrane permeabilization; MCOLN1: mucolipin TRP cation channel 1; mTORC1: mammalian target of rapamycin complex 1; TFEB: transcription factor EB; V-ATPase: vacuolar ATPase.

processes implicated in cellular metabolism and growth. The complexity and broadness of the mTOR signaling networks increase the risk of toxicity due to off-tumor on-target effects, as the therapeutic window is narrow, if existent[99]. Besides, accumulating evidence suggest that mTOR is not the only specific molecular target for rapamycin. A quantitative chemical proteomics approach has revealed that the rapamycin targetome is extensive, including Stat3, an ubiquitous secondary messenger[100]. In consequence, the efficacy of mTORC1 modulators in clinical trials is limited[101-104], probably due to its conserved function of mTOR complex in homeostasis mechanisms in all cell types, preventing their further clinical development [Table 1].

Table 1. Summary of the main lysosome-associated chemoresistance mechanisms and therapeutic approaches

CHEMORESISTANCE MECHANISMS RELATED TO LYSOSOMES			
Mechanism	**Cause**	**Effect**	**Solution**
Drug sequestration[59]	Drug protonation due to lysosomal acidic lumen[64,65]	Changes in subcellular distribution[77]	Increase drug concentration[78-80]
Exocytosis[83]	Drug accumulation due to increased pH[84,85] and TFEB activation[86,87]	Clearance of lysosomal drug content[86,87]	Drugs can rediffuse back into the cells
LYSOSOME-BASED THERAPEUTIC APPROACHES			
Type	**Actions**		**Examples**
Lysosomal destabilizers	Lysosomal membrane permeabilization, cathepsins release, and cell death program activation[92]		Mefloquine (anti-malaria drug)[25], cationic amphiphilic antihistamines[93], and siramesine (σ2 receptor agonist)[94]
V-ATPase inhibitors	Activation of cell death program[96]		Archazolid A[96] and bafilomycin A[98,96]
mTOR modulators	Effect on combinatory therapies[52]		Rapamycin[52]
Antibody-drug conjugates	Release of the therapeutics coupled to the antibody[105]		Gemtuzumab ozogamicin[106]

Gemtuzumab ozogamicin (Mylotarg) is an anti-CD33 monoclonal antibody conjugated to the small molecule chemotherapy drug calicheamicin, recently reapproved for AML. Upon surface CD33 recognition, this antibody-drug conjugate is internalized and translocated to lysosomes. The acidic-labile linker is hydrolyzed in the acidic environment of the lysosome, releasing the cytotoxic drug that is exported to the nucleus, where the pharmacological effect occurs[105]. Thus, the effectiveness of gemtuzumab ozogamicin greatly depends on lysosome functionality. This targeted therapy was expected to represent a new paradigm in AML therapy; however, the clinical benefit is limited and severe adverse effects are found in a considerable rate[106]. Discrepancies between expectations and clinical efficacy may be explained based on the lysosomal impairment in AML cells, partially due to the hyperactivation of PI3K/Akt signaling pathway[51,53,54]. A direct correlation between lysosome function and gemtuzumab ozogamicin-induced cytotoxicity was observed and forced activation of lysosomes led to a synergistic effect with gemtuzumab ozogamicin[106], demonstrating that these lysosomal-dependent conjugate approaches used as monotherapy may be of limited interest in AML.

CONCLUSION

Refractory and relapse disease are still the main clinical challenges faced in AML. Although new drugs have been approved in the last years, treatment failure and resistance mechanisms remain a major problem in patient management. To date, none of the therapeutic strategies designed to overcome chemoresistance has succeeded in clinics. The identification of lysosomes as key organelles in resistance acquisition opened a new research field and provided new avenues to explore in order to revert the resistant phenotype. The leukemic transformation process results in an augmented metabolic demand, associated with the upregulation of the lysosome function. Consequently, increase in lysosomal mass, pH, and enzymatic activity is induced. Lysosomotropism of several chemotherapeutics enable their sequestration in the lysosomes, becoming "drug-safe house" compartments and reducing their cytotoxic effect in molecular targets. As a consequence of the lysosomal changes induced during leukemogenesis, AML lysosomes are more fragile than those found in healthy cells, with a preclinically demonstrated safe therapeutic window. Developing new drugs that target leukemic lysosome integrity may sensitize AML cells to conventional chemotherapeutics or even constitute a new pharmacological lysosome-centred strategy for relapse and refractory AML patients.

DECLARATIONS

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Authors' contributions

Preparing and reviewing the manuscript: Cuesta-Casanovas L, Delgado-Martinez J, Cornet-Masana JM, Carbó JM, Clément-Demange L, Risueño RM

Availability of data and materials

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Conflicts of interest

Cornet-Masana JM, Carbó JM, Clément-Demange L, and Risueño RM are inventors in patents related to acute myeloid leukemia treatments. Risueño RM is a shareholder of Leukos Biotech. Cuesta-Casanovas L and Delgado-Martinez J declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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In vitro cultures of circulating tumor cells: a potential tool to unravel drug sensitivity

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Abstract

Since taking part as leading actors in driving the metastatic process, circulating tumor cells (CTCs) have displayed a wide range of potential applications in the cancer-related research field. Besides their well-proved prognostic value, the role of CTCs in both predictive and diagnostics terms might be extremely informative about cancer properties and therefore highly helpful in the clinical decision-making process. Unfortunately, CTCs are scarcely released in the blood circulation and their counts vary a lot among different types of cancer, therefore CTC detection and consequent characterization are still highly challenging. In this context, *in vitro* CTC cultures could potentially offer a great opportunity to expand the number of tumor cells isolated at different stages of the disease and thus simplify the analysis of their biological and molecular features, allowing a deeper comprehension of the nature of neoplastic diseases. The aim of this review is to highlight the main attempts to establish *in vitro* CTC cultures from patients harboring different tumor types in order to highlight how powerful this practice could be, especially in optimizing the therapeutic strategies available in clinical practice and potentially preventing or contrasting the development of treatment resistance.

Keywords: Liquid biopsy, circulating tumor cells, liquid tumor biomarkers, cell cultures, circulating tumor cell cultures, biomarker evaluation, precision medicine, drug sensitivity

CIRCULATING TUMOR CELLS: WEAKNESS IN RARITY

During the last decades, personalized medicine has progressively gained a crucial role in cancer therapeutics, leading to an increased need to monitor the molecular heterogeneity of neoplastic diseases. Despite representing the gold standard in defining tumor molecular profile and therefore in guiding treatment choices, tissue biopsy is an invasive practice that does not allow to follow the clonal evolution of the tumor, thus being not very informative of the genomic changes that cancer might exhibit at progressive disease[1]. In this context, the increasing interest showed in liquid biopsies together with the consistent number of studies in this research field have led to a deeper consciousness of the potential impact that liquid tumor biomarkers could have on the clinical decision-making process, especially considering that liquid biopsies are non-invasive, easy to perform, and highly accessible[1,2]. As the only requirement to obtain information about the tumor mutational status is a simple withdrawal of biological fluids, most frequently blood but also saliva, urine, pleural, and cerebrospinal fluid, liquid biopsies enable a real-time closer look at cancer dynamics, ensuring a more precise monitoring of cancer patients, hopefully offering the best therapeutic option possible[3].

Circulating tumor cells (CTCs), circulating tumor DNA, and more recently exosomes released from both primary tumor and metastatic sites in the systemic circulation represent the main cancer-derived material studied in liquid biopsy-based analyses, providing prognostic and predictive indications and currently under investigation for their potential role in cancer diagnostics[1,3].

CTCs were firstly described by Ashworth[4] in 1869 and, after only twenty years, Stephan Page proposed the "seed and soil" hypothesis, assuming that the metastatic process is not randomly successful, but tumor cells (the "seed"), dethatching from the primary tumor, can grow up in selected organs solely in the presence of a suitable microenvironment (the "soil")[5]. This theory was not immediately accepted, but only after a century, CTCs were recognized for their relevant role in driving the metastasization[6]. CTCs, shed actively or passively in the bloodstream, can be found as single cells or aggregated in clusters, with clusters appearing to show up to 50-fold increased metastatic potential compared to single CTCs[7,8]. The number of CTCs detected through the antigen-dependent CellSearch® system shows a prognostic value for metastatic breast, colon, and prostate cancers[9-12]. CTCs counts ranging from 0 up to 4 CTCs in 7.5 mL of blood are indicative of a favorable prognosis, while a cut-off of 5 CTCs in 7.5 mL of whole blood indicates an unfavorable prognosis for both metastatic breast and metastatic prostate cancers, while in the case of metastatic colon cancer a cut-off of 3 CTCs suggests poor prognosis[9-11]. Currently, CTC enumeration through CellSearch® for these three metastatic cancer settings is the only clinical application for CTCs[13].

Unfortunately, since CTCs isolation through the CellSearch® platform relies on the expression of the epithelial cell adhesion molecule (EpCAM), which is normally found on the surface of cells of epithelial origin, there may be problems related to CTCs discrimination and detectability because CTCs are actually capable of downregulating epithelial characteristics in favor of a mesenchymal-like phenotype[7,14,15]. Morphological and genetic changes determining this phenotypical switch can be identified in the activation of a biological program termed epithelial-mesenchymal transition[7,16]. The acquisition of a mesenchymal-like phenotype strongly enhances the migratory and invasive capability of tumor cells, therefore the metastatic competence of these cells is incredibly increased[16]. Moreover, this phenotypical plasticity is highly associated with stemness properties as well as drug resistance, and it is mainly responsible for the impossibility of CellSearch® to detect and isolate CTCs[7,14-16].

To overcome this limitation, several alternative devices based on CTCs physical or functional characteristics have been developed over the years[7]. However, the use of these methods is limited to research purposes and

currently does not find any applications in clinical practice.

A major issue to consider when studying CTCs is their rarity in blood circulation[3,6,17]. The scarce formation of CTCs in the body together with their variability in different tumor types represent hard challenges in CTC analysis[3,6,17]. In this context, it is particularly clear the potential usefulness of CTC cultures[17]. *Ex vivo* expansions of CTCs can possibly enable to obtain a conspicuous number of circulating tumor cells and consequently to study tumors characteristics not only present in the primary site, but also defining the properties of cells able to survive in the blood circulation as well as to form distant metastases and eventually understanding the changes occurring between the first stages of the tumor and the advanced ones, thus implementing the use of liquid biopsies[18]. This would be strongly important, for example, in elaborating new therapeutic strategies for cancer patients and concurrently overcoming the problems related to the onset of resistance phenomena[3,19-23]. Despite the huge interest in CTC cultures, only a few groups succeeded in establishing CTC cultures, thus confirming the enormous difficulties that still make this practice extremely challenging today[12].

CTC BIOLOGY

CTCs have a crucial role in tumor progression, taking part in the intermediate stage of the process known as metastatic cascade[24]. Indeed, the metastatic evolution of cancer is a sum of several events, starting with tumor cells dissemination from the primary site of the neoplastic mass (or alternatively from an already formed metastatic lesion), followed by a phase of invasion directly into the blood vessels or indirectly passing through the lymphatic circulation, ultimately concluding with the colonization of a distant organ and the new tumor formation[25,26].

CTCs intravasation can occur both through active and passive shedding[26]. Cell-intrinsic features, microenvironmental characteristics, and vascular structure could be involved in the generation of CTCs[26]. The activation of the epithelial-mesenchymal transition program, guided by a group of different transcriptional factors (e.g., Snail, Slug, Twist, and Zeb1), leads tumor cells to lose cell polarity, acquire the capability to degrade components of the extracellular matrix, and downregulate such epithelial characteristics like EpCAM and E-cadherin surface proteins (extremely important for cell-cell adhesion) in favor of mesenchymal-like features, together with the invadopodia formation regulated by the N-WASP protein, concur to the active release of cancer cells in the bloodstream[25-27]. The presence of a leaky vascular structure, highly associated with tumor growth, can contribute to the generation of CTCs[26]. The chronical activation of angiogenesis is notably one of the hallmarks of cancer[28]. The dysregulation of proangiogenic signals, like the fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF), promotes the formation of aberrant vessels, thus facilitating both the passive shedding of cancer cells and the active intravasation[26,28]. Lastly, the tumoral microenvironment plays a key role in inducing the formation of CTCs[26]. Interestingly, it has been reported that tumor-associated macrophages, expressing the tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains-2 (Tie-2), are capable of producing VEGF, therefore contributing to the increase of vascular permeability and ultimately to tumor cells intravasation[29].

Once in circulation, it is possible to find CTCs both as individual cells and clusters of 2 to 50 cells[26]. Several studies regarding CTC clusters detection, their characteristics and functional role have been published during the last decade. Particularly interesting is the work of Aceto *et al.*[30] demonstrating that CTC clusters derive from oligoclonal groups of cells arising from a single tumor, excluding cluster formation by coalescence of single CTCs in the bloodstream. This study ulteriorly reported the half-life of CTC clusters, ranging from 6 to 10 min, thus considerably shorter compared to single CTCs (estimated to be 25 to

30 min)[30]. The reduced half-life of CTC clusters, together with the ability to extravasate faster than single CTCs and the enhanced metastatic competency (partly associated with resistance to apoptosis), support their survival and outgrowth[30].

In 1975, Butler and Gullino[31] quantified the rate of tumor cells shedding in the bloodstream using a rat model. This study showed that tumors release millions of cancer cells in the blood circulation per 24 h/g of tissue[31]. Despite the huge amount of tumor cells shed each day, patients commonly develop only few metastases, thus demonstrating the metastatic process as highly inefficient[32]. Indeed, the cell viability in the blood is compromised by several factors, including shear stress, *anoikis*, the deficit of growth factors, and immune surveillance[32,33]. The combination of such aspects results in a tremendously limited number of CTCs, commonly 1 to 10 CTCs per mL of blood[33,34].

Although many factors concur in reducing the survival of CTCs in the circulation, important evidence indicates various blood constituents, including platelets, neutrophils, macrophages, myeloid-derived suppressor cells (MDSCs), or cancer-associated fibroblasts to tightly interact with CTC preserving them from physical damage and helping them in evading the immune system[35]. Platelets are actively involved in CTCs' protection during their transit into the bloodstream in many ways, for example, defending them from mechanical stress, as well as cancer-associated fibroblasts, and inducing resistance from *anoikis*, which is regulated by the YAP pathway, determining RhoA-(myosin phosphatase targeting subunit 1) and MYPT1-protein phosphatase (PP1)-mediated Yes-associated protein 1 (YAP1) dephosphorylation and nuclear translocation[35,36]. Most importantly, platelets can recruit either neutrophils and macrophages by the release of chemokines, like CXCL5 or CXCL7[37]. Together with neutrophils, platelets can shield tumor cells from other immune cells attacks, thus helping CTCs in immune escaping[35,37]. Similarly, CTC-MDSC clusters seem to be capable of evading T cell immune response[35]. Moreover, interactions with blood cells can significantly enhance the ability of those CTCs to invade distant sites. It has been reported that platelets can release transforming growth factor β (TGFβ), known to be involved in the activation of the epithelial-mesenchymal transition program, thus incrementing the invasion potential of CTCs, and other mediators, like histamine, serotonin, and eicosanoid metabolites among others, determining the modification of blood vessel permeability[37-39]. On the other hand, cancer cells releasing colony-stimulating factor 1 can activate macrophages, which, in turn, secrete epidermal growth factor, therefore inducing tumor cell migration[40].

Complex interactions with blood constituents combined with the expression of specific genetic signatures, which seem to be associated with the ability of CTCs to metastasize (e.g., in a study by Zhang *et al.*[41], breast cancer CTCs expressing the Her2+/EGFR+/HPSE+/Notch1+ genetic signature demonstrated a tendency to metastasize in the brain), allow an extremely small fraction of cancer cells in the bloodstream to complete the metastatic cascade, thus colonizing distant tissues and promoting the generation of new tumor lesions.

Hence, it is clear that CTCs with metastatic competence are definitely rare events, delineating the metastatic cascade as a globally inefficacious process. Furthermore, although it is possible to count a large number of works elucidating the main characteristics of CTCs, there are lots of questions yet unraveled. Futures studies, providing a deeper knowledge of the biology of these circulating cells, are needed to consolidate the clinical value and the use of CTCs, particularly in the field of precision medicine.

CTC CULTURES: A STRENUOUS CHALLENGE

Several strategies are currently available for CTCs isolation and subsequent culturing. The enrichment is a crucial step when it comes to isolating viable CTCs from the rest of the blood constituents, such as platelets, red blood cells and white blood cells, allowing CTCs concentration and thus facilitating the detection

process[12,17]. It is possible to perform the enrichment step through three different types of technologies: protein expression-based, physical property-based and function-based technologies[12,17] [Figure 1].

In the first case, the enrichment feasibility is due to the expression or the lack of the expression of specific proteins on the surface of CTCs. In other terms, CTC isolation relies on the presence of certain positive or negative selection markers[12,17]. EpCAM is one of the most frequently used surface proteins for positive enrichment, while surface antigens, like CD45, serve to guarantee the depletion of leukocytes from the blood sample by negative selection[12,17]. Among the positive selection-based techniques, immune-magnetic separation, flow cytometry and immuno-affinity based microfluidic platforms, like the CTC-iChip, must be mentioned[12]. The drawback of these methods is strictly related to the possible loss of CTCs during the isolation, due to the lack of expression of the antigens selected for the positive enrichment[12,17]. To overcome this problem, CTCs can alternatively be separated by negative selection. RosetteSep® antibody cocktail, for example, is an available product currently used to enrich CTCs through tetrameric complexes of antibodies that target the unwanted cells in the blood sample, allowing their removal and eventually CTC purification[12]. These tetrameric antibody complexes recognize several antigens, including CD2, CD16, CD19, CD36, CD38, CD45, CD66b, and glycophorin A on red blood cells and leukocytes[42]. After centrifugation using a density gradient medium, purified tumor cells can be found at the interface between the plasma and the medium[42].

Since CTCs are supposed to be larger and less deformable than other blood cells (even though recent studies showed the existence of small CTCs potentially implicated in metastasis progression) and have different relative densities compared to red blood cells and white blood cells[43], separation based on the physical properties play a leading role in the field of CTC enrichment[12,17]. Indeed, it has been generally reported that CTCs exhibit an average size of 30 μm, bigger than red blood cells (6-8 μm) and white blood cells (10-15 μm)[44,45]. Thus, several size-based platforms have been developed to isolate CTCs, such as MetaCell®, as well as different microfluidic devices, like the Parsortix®[12]. While MetaCell® enables CTC enrichment through an 8 μm-pore polycarbonate membrane, Parsortix® is a microchip-based isolation technique[46-50]. Both methods give the possibility to isolate viable CTCs[12], with MetaCell® also being frequently used to establish short-term CTC cultures[46-50]. Moreover, in 1959, Seal, observing that red blood cells and white blood cells have higher specific gravities (1.092 and 1.065, respectively) compared to cancer cells (1.056), exploited these differences to separate them using one of the first density-based techniques involving silicone floatation[51]. To date, it is possible to count on different density gradient media, such as Ficoll®, a synthetic polymer formed by copolymerization of sucrose and epichlorohydrin[12], Ficoll-Paque® and Lymphoprep®, both composed of polysaccharides and diatrizoate[52], and Percoll®, consisting in a colloidal silica particle suspension[45]. All these media basically allow the separation of CTCs from blood cells through centrifugation. To optimize this type of CTC enrichment, density gradient media are frequently used with other methods, like the aforementioned RosetteSep® antibody cocktail[53].

Eventually, another opportunity to enrich viable CTCs is offered by the isolation of tumor cells based on their functional properties[12,17]. Almost ten years ago, the development of a method allowing CTCs enrichment by their invasion ability, known as collagen adhesion matrix assay, represented the first example of this methodological category[17]. Essentially, this assay is based on the singular propension of tumor cells to disrupt and ingest collagen adhesion matrix fragments, providing information about cancer cells invasiveness[54]. On the other hand, the detection of proteins secreted, released or shed by viable cancer cells, is the core of the EPithelial ImmunoSPOT (EPISPOT) assay[55]. These types of techniques enable *in vitro* CTC expansions since they well preserve cell viability[12,17] [Table 1].

Table 1. Examples of strategies for viable circulating tumor cell (CTC) isolation

Type of technology	System	Method	Type of culture	Ref.
Protein expression-based technologies	RosetteSep®	Antibody cocktail for negative selection	Short-term Long-term	Guo *et al.*[12]
	CTC-iChip	Micro fluidic capture platform with two immune magnetic sorting modes to isolate CTCs	Long-term	Yu *et al.*[19]
Physical property-based technologies	Ficoll® Ficoll-Pacque® Lymphoprep® Percoll®	Density gradient media that allow the separation of circulating tumor cells from blood cells through centrifugation	/	Guo *et al.*[12] Li *et al.*[45] Rosado *et al.*[52]
	MetaCell®	8 μm-pore polycarbonate membrane-based technique.	Short-term	Kolostova K. *et al.*[46]
	Parsortix®	Cell size and deformability microchips isolation based technique	/	Guo *et al.*[12]
Function-based technologies	Collagen adhesion matrix assay (CAM)	Method based on tumor cells' ability to attach and ingest collagen adhesion matrix	/	Guo *et al.*[12]
	EPithelial ImmunoSPOT assay (EPISPOT)	Detection of proteins secreted or released by viable cancer cell	Short-term	Guo *et al.*[12]
			Long-term	Cayrefourcq *et al.*[62]

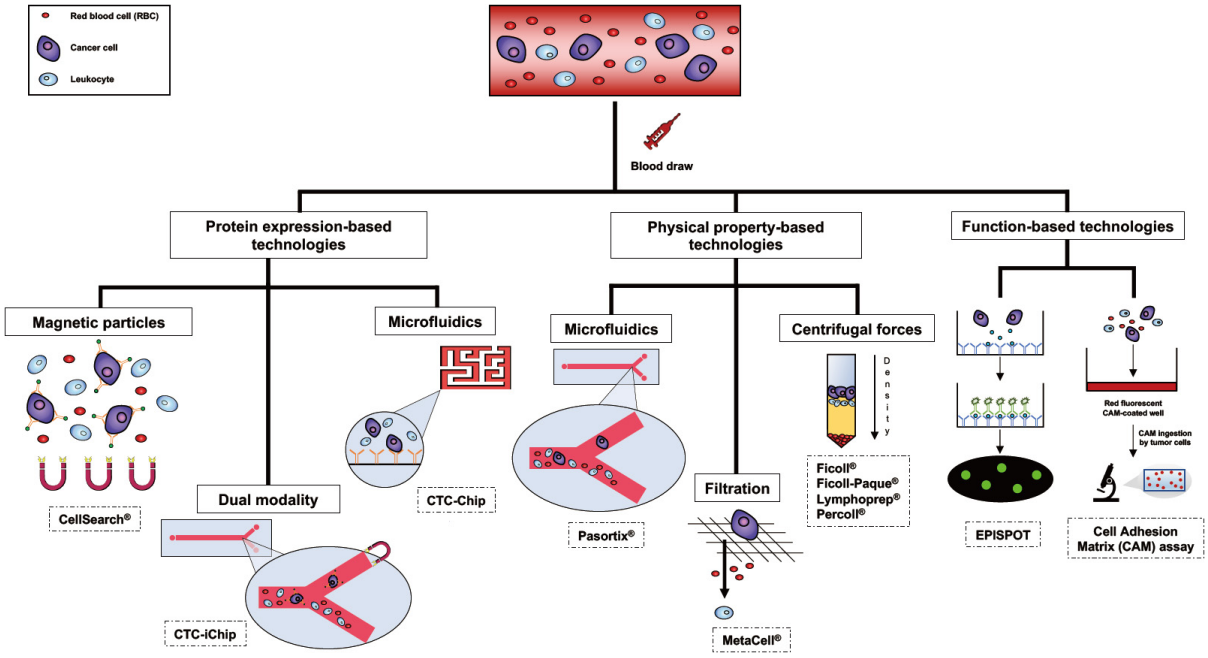

Figure 1. Schematic examples of the most commonly used technologies for circulating tumor cell (CTC) enrichment, detection, and isolation.

Despite the numerous methods currently available for CTCs enrichment and therefore the resulting complexity in selecting a more suitable technique to enrich viable CTCs for future cultures, the choice of the culturing conditions is probably the hardest part of the *in vivo* expansion process. In this context, the critical challenge is related to the limited knowledge we have about the biology of the CTCs[12] and, most of all, the prerogative of CTCs to show a high rate of heterogeneity[14] that overcomplicate the adoption of certain culturing options and is consequently decisive in succeeding or failing the *ex vivo* CTC propagation[12]. According to the scientific literature, it is possible to count on a discrete number of studies reporting examples of CTCs *in vitro* cultivations. In 2013, Zhang *et al.*[41] reported for the first time an *ex vivo* expansion of CTCs for breast cancer, also demonstrating those CTCs to have metastasis initiating properties

in the brain when expressing a specific genetic signature (the aforementioned Her2+/EGFR+/HPSE+/Notch1+). For this study, CTC cultures were monitored over 28 days, therefore it was not possible to establish long-term cultures[41]. Comparable cultures of circulating cancer cells were obtained from patients with mesothelioma[56], lung[57], esophageal[58], bladder[59], and head and neck cancers[60]. In all these cases, CTCs were efficiently maintained in culture for a short period (in most cases, 14 days, with rare exceptions, which, however, did not exceed 50 days of cultivation)[56-60]. Only a few groups successfully obtained long-term CTC cultures, including Yu *et al.*[19] and Gao *et al.*[61] in 2014, respectively for breast and prostate cancers. Additionally, Cayrefourcq *et al.*[62] in 2015, Grillet *et al.*[21] in 2017, and Soler *et al.*[63] in 2018 were able to establish durable CTCs cultures and permanent CTC lines from colorectal cancer patients[62,63]. Brungs *et al.*[22] obtained long-term CTC cultures in 2020 for metastatic gastroesophageal cancer, while Hamilton *et al.*[20] in 2015 and Lee *et al.*[23] in 2020 obtained successful CTC cultures from small cell lung cancer (SCLC) patients.

The two main strategies adopted to establish *in vitro* circulating cancer cell cultures are represented by two-dimensional (2D) and three-dimensional (3D) cultures[12]. Adherent conditions are the most common choice, especially for short-term cultures, because it is clearly easier to set up a 2D culture, in terms of time, complexity, and costs[12,64]. However, even if there are studies reporting data of successful short-term and long-term CTC cultures in both 2D and 3D systems, it has been observed that a non-adherent culturing condition is preferable when the aim is to establish long-term cultures, because following a few cell divisions, CTCs cultured according to a monolayer adherent approach tend to senesce[19]. Besides this, when cultured in 2D conditions, CTCs actually lose essential morphological characteristics as well as cell-cell and spatial interactions, thus several cellular functions, like proliferation or differentiation, are definitively compromised[64]. Moreover, the adherent culture condition guarantees unlimited access to nutrients and oxygen, in contrast with the actual *in vivo* situation[64]. Different studies reported the importance of hypoxic conditions in promoting CTCs growth[19,62], while other publications showed the capability of CTCs to grow even under a non-hypoxic context[61].

Although recreating the exact characteristics of a tumor microenvironment is almost impossible, there have been many successful attempts in culturing CTCs as well as mimicking the *in vivo* tumor growth, for example, co-cultures of CTCs from early lung cancer patients together with tumor-associated fibroblasts, collagen I, and Matrigel[57].

Alternatively, *ex vivo* expansions of CTCs can be implemented by direct inoculation of CTCs in immunodeficient mice. Major brilliant examples of CTC-derived explants were published during the last two decades, starting with the work of Pretlow *et al.*[65] in 2000, where the authors reported the capacity of cells in peripheral blood of prostate and colorectal cancer patients to form metastasis. In 2013, Baccelli *et al.*[66] demonstrated the existence of a population of metastasis-initiating cells among breast cancer CTCs that express EPCAM, CD44, CD47, and MET, thus recapitulating the phenotype of patient metastases. Lastly, Hodgkinson *et al.*[67] elegantly described that CTCs isolated from SCLC patients, either showing sensitivity or resistance to chemotherapy, then inoculated into immune-compromised mice were tumorigenic and mirrored the donor patient's response to treatments. It is important to point out that the feasibility of these *in vivo* assays is associated with the presence of very high concentrations of CTCs in the blood samples (e.g., > 1000 in 7.5 mL of peripheral blood in the case of breast cancer patients), therefore reducing the possibility to successfully realize CTC-derived explants[62].

However, *ex vivo* propagations of cancer cells are still extremely delicate processes, therefore more and more studies are needed to optimize all the steps involved in this practice.

CTC MOLECULAR CHARACTERIZATION: DIVING INTO PRECISION MEDICINE

Cancer is definitely not a static disease, therefore spatial and temporal tumor dynamics must be considered when analyzing the neoplastic evolution to guide patients towards the best clinical outcome possible[68]. Since treatment decisions solely rely on primary tumor sampling, there clearly could be a massive loss of information about tumor characteristics that would be otherwise important in the therapeutic evaluation process, especially considering that treatments are actually directed against metastases rather than primary tumors[68,69]. Indeed, the selective pressure deriving from both the metastatic process and microenvironmental features can induce the formation of subclones presenting properties that highly differ from the primary tumor[7]. Thus, the analysis of metastatic cells is crucial for clinical practice purposes[7]. However, in several cases (e.g., lung or brain metastases), tissue sampling is certainly not feasible[7]. Given that metastatic cells can be constitutive of the pool of CTCs, liquid biopsy can definitely provide a valid alternative to this invasive practice[69].

The molecular characterization of CTCs might offer details about the changes occurring during the metastasization process and possibly elucidate the reasons for resistance to therapy as well as highlight potential therapeutic targets[69]. In breast cancer, for example, human epidermal growth factor receptor 2 (HER2) positive CTCs were found in patients with HER2-negative primary tumors suggesting the proper therapeutic regimen based on the HER2-CTC status[70-72]. Comparably, patients with estrogen receptor (ER) positive primary tumors were found to be characterized by the presence of ER-negative CTCs[73]. This may predict the onset of resistance to endocrine treatment in the subpopulation of metastatic breast cancer patients who do not benefit from this therapy[7,73]. Other analyses of CTCs at the single-cell level showed to be informative of the estrogen receptor 1 (*ESR1*) gene mutations, thus allowing the identification of genes involved in endocrine treatment resistance[74]. In metastatic castration-resistant prostate cancer (mCRPC), an androgen-dependent type of prostate cancer, the cause of a failing response to androgen receptor (AR) inhibitors, enzalutamide and abiraterone, can be identified in the presence of androgen-receptor splice variants[75]. Androgen-receptor splice variant 7 (AR-V7) was actually found to be expressed on the surface of CTCs isolated from mCRPC patients, thus confirming the association between the detection of AR-V7 in CTCs and the exerted resistance to AR inhibitors in mCRPC patients[75]. In a study focused on colorectal cancer, genomic analyses conducted on individual CTCs showed the presence of both Kirsten rat sarcoma viral oncogene homolog (KRAS) mutated and KRAS wild-type CTCs in the same patient[76]. Since the expression of KRAS mutations hinder the use of anti-epidermal growth factor receptor (EGFR) treatments, the presence of CTCs harboring KRAS mutations in wild-type colon cancers might explain the therapeutic EGFR inhibition failure, thus being indicative of treatment resistance[7,77,78]. Lastly, programmed cell death ligand 1 (PD-L1) expression on CTCs in metastatic lung cancer seems to be predictive of a good response to anti-PD-1 immunotherapy, so it could potentially become a helpful biomarker when evaluating the response to immunotherapies in the context of precision medicine[79].

IN VITRO CTC CULTURES AS A MODEL TO DECIPHER DRUG SENSITIVITY

Considering the low concentration of CTCs in the bloodstream as well as their high rates of heterogeneity and poor survival, not surprisingly, there are still a lot of open questions about CTC biology, including the timing of their release in the blood, the extravasation and intravasation processes, their ability to survive once in the systemic circulation and which is the genetical relation with the original tumor. Moreover, despite their identification and study in different neoplastic diseases, the aforementioned issues (first and foremost, the poor number of CTCs released in the blood) represent remarkable obstacles to CTCs characterization at genomic, transcriptomic, and functional levels. Therefore, *in vitro* culturing of CTCs can offer the opportunity to overcome these limitations and better elucidate the molecular features of these cells.

Unfortunately, CTCs are definitely not easy to expand *in vitro*, especially when the purpose is to establish long-term CTCs cultures[8,12,80,81]. However, for those groups who succeeded in culturing CTCs, it was possible to observe that these cell lines they obtained show phenotypical characteristics that partially match those of cells present in tumor tissues from donor patients, but also express molecular features specifically related to CTCs[19,41,62,82], exhibiting metastatic competency[66,67] as well as stemness properties including an efficient DNA repair system and an enhanced metabolic rate[82]. Furthermore, since being representative of the tumor molecular landscape, CTC cultures can be useful in predicting drug sensitivity as well as treatment resistance and eventually became a precious tool in drug screening projects[83] [Figure 2].

In a ground-breaking study, dated 2014, Yu *et al.*[19] isolated viable CTCs, using the CTC-iChip technology, and successfully established long-term oligoclonal CTC cultures from six ER-positive breast cancer patients and sustained these cultures for over six months. The analysis of these cell cultures allows exploration of the unique genetic context of single tumors, thus revealing the presence of preexisting mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA) gene and the acquisition of new tumor-derived mutations in *ESR1* gene, *PIK3CA* gene, and fibroblast growth factor receptor gene 2 (*FGFR2*), among others[19]. Furthermore, the CTC lines obtained were used for drug sensitivity testing, they resulted concordant with patients' clinical histories, and they were also useful for the identification of new potential therapeutic targets[19]. More specifically, one of these cell lines, displaying a high allele frequency of mutant ERS1, was actually not sensitive to selective ER modulators and the selective ER degrader fulvestrant[19]. However, since ER strongly depends on the activity of HSP90 protein and the stabilization of mutated receptors relies on this chaperone, the use of HSP90 inhibitor in this ERS1 mutated cell line was found to be cytotoxic alone and in combination with both raloxifene and fulvestrant[19]. In another CTC line, harboring both *PIK3CA* and *FGFR2* gene mutations, the combined inhibition of these two targets (not previously tested in clinical settings) showed to successfully enhance the activity of single drugs[19] [Table 2].

Hamilton *et al.*[84], who firstly obtained stable CTCs cultures from SCLC patients in 2015, later in the same year conducted a drug sensitivity study using two CTCs lines, BHGc7 and BHGc10, that were established from peripheral blood of SCLC patients with the extended disease[20]. Since CTCs counts are monitored for prognostic purposes and to evaluate response to cytotoxic therapy, they treated SCLC BHGc7 and BHGc10 CTC cell lines with common second-line therapies for SCLC (cisplatin, etoposide, topotecan, and epirubicin) *in vitro* and compared the chemosensitivities of these cell lines to drug responsiveness of several permanent SCLC cell lines derived from lung and distinct metastases[20]. The cytotoxicity assays showed that BHGc10 was way more resistant than BHGc7 to cisplatin, while the other SCLC cell lines exerted variable responses to this chemotherapeutic agent[20]. Both BHGc7 and BHGc10 were sensitive to etoposide as well as two SCLC cell lines[20]. The remaining SCLC cell lines displayed elevated IC_{50} values instead[20]. Either BHGc7 and BHGc10 were found to be highly sensitive when treated with topotecan or epirubicin[20]. Compared to the CTC cell lines, SCLC cell lines exhibited different susceptibilities to epirubicin and topotecan, showing a significant resistance to epirubicin, while milder to topotecan (with the exceptions of topotecan-sensitive SCLC26A cell line and, conversely, NCI-H526 distinctly topotecan-resistant)[20]. The heterogeneous responses displayed by CTCs in comparison to primary and metastatic SCLC cells not only highlight the intrinsic difference in terms of drug sensitivities among these cancer cells, but also clearly indicate that the variation in CTCs number can possibly not mirror the response of primary and metastatic SCLC lesions to chemotherapeutic treatment[20] [Table 2].

Later in 2017, Grillet *et al.*[21] established three distinct CTC cell lines by isolating and expanding CTCs from chemotherapy-naïve patients with metastatic colorectal cancer. These cell lines, cultured for several months, showed up to have strong cancer stem cell phenotypical traits, with self-renewal and multilineage

Table 2. Patients involved, CTC lines obtained and drug sensitivities

Authors	Type of tumor	*n* patients involved	*n* of CTC cell lines obtained	CTC lines name	Molecular signatures	Sensitivity
Yu *et al.*[19]	Metastatic luminal subtype breast cancers	36	6	BRx-33	*ESR1*, *NUMA1*	/
				BRx-07	*TP53*, *PIK3CA*, *FGFR2*, *CDH1*, *APC*, *DGKQ*, *MAML2*	Paclitaxel*, fulvestrant* and doxorubicin* *FGFR2* inhibitor AZD4547 *PIK3CA* inhibitors (BYL719 and PD173074) Moderately responsive to the *FGFR1* inhibitor PD173074
				BRx-68	*TP53*, *ESR1*, *PIK3CA*, *MSN*	Capecitabine*, fulvestrant* *HSP90* inhibitor STA9090 alone and in combination with both raloxifene and fulvestrant
				BRx-50	*ESR1*, *IKZF1*, *BRCA2*	Capecitabine*, olaparib*
				BRx-42	*PIK3CA*, *KRAS*, *IGF1R*	/
				BRx-61	*TP53*	/
Hamilton *et al.*[20]	Small cell lung cancer	2	2	BHGc7	/	Cisplatin, etoposide, topotecan and epirubicin
				BHGc10	/	Etoposide, topotecan and epirubicin Mild resistance to cisplatin
Grillet *et al.*[21]	Metastatic colorectal cancer	/	3	CTC41 CTC44 CTC45	*BRAF V600E*, CSC-related genes (*ALDH1A1*, *CD133*, *CD26*, *CD44v6*)	Resistant to FIRI (regimen inspired by standard-of-care chemotherapy combinations 5-fluorouracil and SN-38, the active metabolite of irinotecan)
Brungs *et al.*[22]	Metastatic gastroesophageal cancer	20	1	UWG01CTC	Genes for neuroendocrine markers (*CNTFR, PAX-5, NGF*), *DLL-3*	Synergism at all concentrations of carboplatin and etoposide Carboplatin increase radiosensitivity
		41	1	UWG02CTC	*Helicobater pylori* mediated carcinogenesis genes (*AKT24*, *ETS225*, *MYC*), *CDH1*, CSC-related genes (*CD44*, *ALDH1*, *CD133*), *EGFR FGFR2*, *HER-2*, *MET*, DNA repair kinases (*ATM* and *ATR*), notch ligand delta-like ligands (*DLL-1* and *DLL-4*), *PLA2GA*, *JAK/STAT* pathway genes	30-40× more sensitive to doxorubicin and etoposide than UWG01CTC
Lee *et al.*[23]	Small cell lung cancer	22	18	/	*TTF-1*, synaptophysin, *PD-L1*, variable expression of EMT markers (E-cadherin, N-cadherin)	CTC lines from patients 14 and 20 showed high sensitivity to standard treatment for SCLC patients (cisplatin/etoposide), whereas these drugs exhibited no cytotoxicity in the CTC line from patient 15, reflecting patient lack of response to this therapy

*Sensitivity to these drugs was consistent with clinical history of the patients. CTC: Circulating tumor cells; EMT: epithelial-mesenchymal transition; FIRI: fluorouracil and irinotecan; FGFR 1 and 2: fibroblast growth factor receptor 1 and 2; HSP90: heat shock protein 90; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

differentiation properties as well as metastatic potential and a robust expression of typical cancer stem cell markers, ranging from aldehyde dehydrogenase (ALDH1A1) and CD133 to CD26 and CD144V6[21]. Since being genetically heterogeneous is another prerogative of these CTC lines, it has been demonstrated

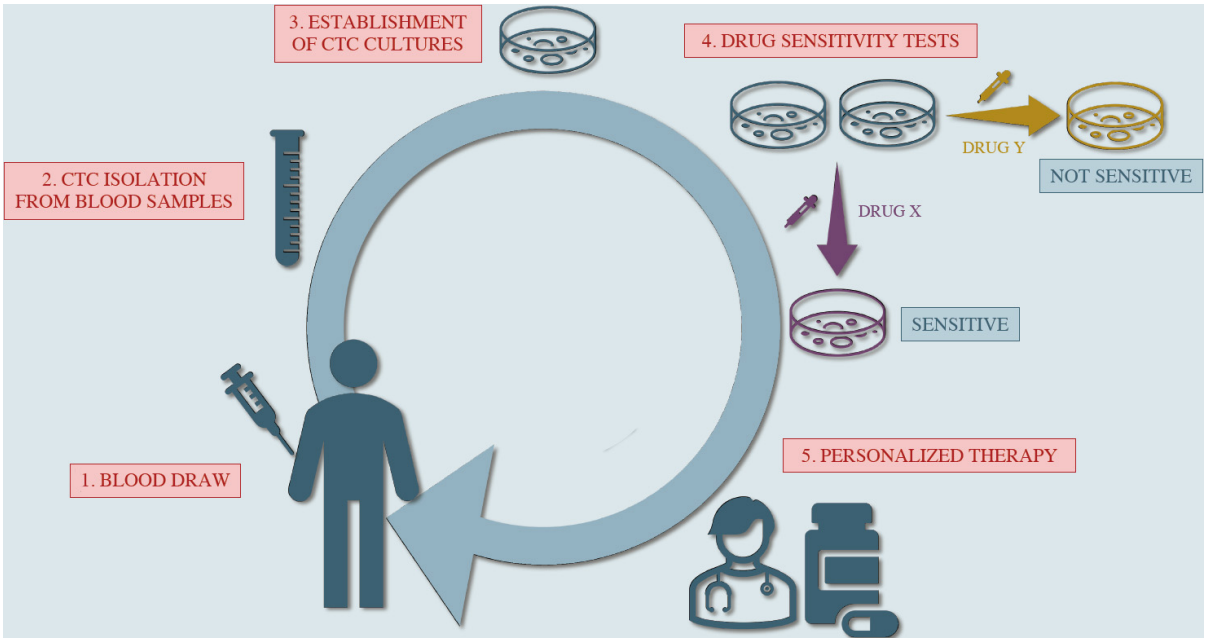

Figure 2. Potential use of circulating tumor cell (CTC) cultures as a predictor for drug sensitivity/resistance, guiding to a more personalized type of therapy.

that they surprisingly harbor the BRAF V600E mutation, although the primary tumors as well as metastatic tissues carried KRAS mutations[21]. Additional analyses provided data highlighting the differential upregulation of metabolic activity in CTC lines compared to primary tumor-derived cells, with a special regard for the enhanced drug/xenobiotics metabolism, thus suggesting these cells to be strongly resistant to standard cytotoxic compounds[21]. These findings were confirmed by following drug sensitivity tests, using a chemotherapy regimen (FIRI: 5-fluorouracil and the active metabolite of irinotecan) that basically mimics the standard treatment combinations for colorectal cancer patients[21]. CTC lines demonstrated more resistance to this therapeutic combination than cells derived from both primary and metastatic tumors[21]. Lastly, evaluating the multikinase inhibitor regorafenib and the BRAF V600 inhibitor vemurafenib activity on these cell lines, a variable sensitivity to regorafenib was exerted and specifically one of these CTC lines emerged to be responsive to vemurafenib, although this BRAF V600 inhibitor demonstrated a scarce efficacy in BRAF-mutated colorectal cancer patients[21] [Table 2].

More recently, a study by Brungs *et al.*[22] reported the establishment of long-term CTC cultures (maintained for over 12 months), with CTCs isolated from metastatic gastroesophageal cancer patients. Profiling the two CTC lines obtained (UWG01CTC and UWG02CTC), data showed these cell lines to display distinct genotypic and phenotypic features, basically reflecting the characteristics of the originating tumor[22]. As the first CTC line (UWG01CTC) was obtained from a patient whose gastrointestinal cancer rapidly developed into metastatic disease, with metastasis histopathology showing high-grade neuroendocrine carcinoma, UWG01CTC exhibited high levels of neuroendocrine markers (i.e., synaptophysin, CD56, and chromogranin A) and strongly expressed genes encoding for neuroendocrine markers such as the ciliary neurotrophic factor receptor (CNTFR), the B-cell-specific activator protein (PAX-5), and the nerve growth factor receptor (NGFR), although this line was negative when stained for epithelial (e.g., EpCAM) or stem cell markers[22]. On the other hand, the patient whose CTC sampling generated the UWG02CTC line was affected by a gastric adenocarcinoma, thus robustly expressing cytokeratins (CK), in particular CK-20, as well as EpCAM and E-cadherin proteins, but also gastric cancer stem cell markers CD44, ALDH1, and

CD133, and demonstrating stem cell pathways, like NOTCH and WNT, to be upregulated[22]. Considering the differences these two CTC lines exhibited, drug and radiosensitivity were evaluated[22]. Indeed, UWG01CTC and UWG02CTC responded differently to standard chemotherapeutics used for these cancers[22]. Moreover, harboring distinct molecular landscapes, with UWG02CTC showing higher expression of EGFR, FGFR2, receptor tyrosine-protein kinase erbB-2 (ERBB2), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway genes compared to UWG01CTC, while UWG01CTC having marked expression of the *DLL3* gene, this study highlights potential druggable targets whose activity should be explored in order to define new personalized types of treatment[22]. Finally, since UWG01CTC displayed lower expression of DNA damage response enzymes than UWG02CTC, it has also been noted that a synergistic effect of radiotherapy when combined with carboplatin for UWG01CTC line[22] [Table 2].

Later in 2020, Lee *et al.*[23] succeeded in expanding *ex vivo* CTCs from SCLC patients. For this study, a new system was developed to culture CTCs, involving the implementation of a biomimetic material called binary colloidal crystal[23]. With the use of binary colloidal crystal, it was possible to build a suitable surface for CTCs expansion, and it was also found that CTC from SCLC formed spheroids, which were observed after 14 days and continued to grow, showing to be still viable after 40 days[23]. During this period, drug sensitivity tests were performed on these CTC cultures to evaluate the response to standard first-line treatment of SCLC, which basically consists of a platinum doublet: cisplatin or carboplatin combined with etoposide[23]. This work demonstrated that CTC cultures recapitulate their originating patients' outcomes, thus highlighting the possibility for expansions of CTCs to predict responses to therapy[23]. Since PD-L1 expression has been detected through immunofluorescence-based analyses, this study also suggests exploring the efficacy of immune checkpoint blockade, which are emerging as a valid treatment for SCLC patients[23] [Table 2].

CONCLUSIONS

Ex vivo expansions of CTCs clearly represent a potential tool to examine tumor characteristics, unraveling new biomarkers as well as possibly predicting drug sensitivity, eventually leading to optimized and personalized treatment strategies. However, the establishment of CTC lines is still tremendously challenging and currently not capable of informing clinical decisions.

One of the most important limiting factors is the timing of the process, which is not rapid enough to guide therapeutic choices for donor patients[3,83]. Indeed, the establishment of CTC lines and their consequent stabilization generally require months to be obtained[3,12]. In this context, several techniques are currently under investigation, such as innovative culture media or support surfaces capable of promoting rapid cell growth and survival[3].

Secondly, since being an essential prerequisite to successfully yield cell lines from CTCs, high CTC counts (> 300 CTCs) constitute another important obstacle that restricts the use of these models in patients with the advanced-stage disease[83]. Novel applications of diagnostic leukapheresis[85] or *in vivo* CTC capture devices[86,87], allowing the isolation of higher numbers of CTCs than conventional enrichment methods, are expected to be a solution for this impediment.

Furthermore, experimental studies suggested that < 0.01% of cancer cells are supposed to initiate metastasis[88-90]. The low frequency of metastatic-inducing CTC among highly heterogeneous populations of tumor cells released in the circulation must be considered particularly when performing drug sensitivity or drug screening tests for clinical purposes[32]. Indeed, as Hamilton *et al.*[20] demonstrated, CTCs may not

reflect treatment responses of primary and metastatic lesions. Moreover, it is crucially important to assess whether the analyses conducted on a cell population that basically derives from few CTCs can be significantly representative of the entire tumor complexity[3].

In conclusion, despite being extremely promising, the use of CTC lines, which will hopefully fulfill soon the great expectation of providing the exact information to offer the best therapeutic option possible to cancer patients, still requires further optimizations to allow translation into the clinic.

DECLARATIONS

Authors' contributions

Draft written, performed data acquisition, as well as provided administrative, technical, and material support: De Renzi G, De Marco G, De Meo M, Del Rosso E, Gazzaniga P, Nicolazzo C

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Original Article

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Circular RNA drives resistance to anti-PD-1 immunotherapy by regulating the miR-30a-5p/SOX4 axis in non-small cell lung cancer

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Abstract

Aim: Circular RNAs are widely and abnormally expressed in human cancer cells, and they participate in cancer progression. However, they have rarely been investigated in the immune evasion of non-small cell lung cancer (NSCLC). Here, we elucidated the function and molecular mechanism of hsa_circ_0020714 in promoting the resistance to anti-PD-1 immunotherapy of NSCLC.

Methods: The expression of hsa_circ_0020714 were examined by qRT-PCR. *In vivo* experiments were executed to investigate the biological function of hsa_circ_0020714 in the sensitivity of NSCLC to anti-PD-1 immunotherapy. The qRT-PCR, fluorescence *in situ* hybridization, RNA pulldown, RNA immunoprecipitation, and western blot were carried out to investigate the potential regulatory mechanisms of hsa_circ_0020714 in NSCLC immune evasion.

Results: The expression of hsa_circ_0020714 was upregulated in NSCLC tissues compared to the paired adjacent non-tumor tissues, and an increased expression of hsa_circ_0020714 was significantly associated with a bad prognosis and resistance to anti-PD-1 immunotherapy in patients with NSCLC. Mechanistically, hsa_circ_0020714 functions as an endogenous miR-30a-5p sponge to enhance SOX4 expression, thereby promoting immune evasion and anti-PD-1 resistance in NSCLC patients.

Conclusion: Hsa_circ_0020714 induces the immune evasion and resistance to anti-PD-1 immunotherapy of NSCLC via the miR-30a-5p/SOX4 axis, and may be an promising immunotherapeutic target in NSCLC.

Keywords: NSCLC, immune evasion, circRNAs, PD-1, immunotherapy resistance

INTRODUCTION

Advanced non-small cell lung cancer (NSCLC) with a negative driver gene has a high tendency for distance metastatic and resistance to chemotherapy[1,2]. Recent evidence suggests a critical role for anti-programmed death-1 (anti-PD-1) immunotherapy in driver gene negative NSCLC. Although anti-PD-1 immunotherapy has improved the prognosis of patients with advanced NSCLC, a larger proportion of patients still did not respond to anti-PD-1 therapy due to primary or secondary resistance and failed to demonstrate clinically effective responses[3,4]. Therefore, further investigation is necessary to clarify the molecular mechanisms involved in NSCLC immune evasion and develop new immunotherapeutic approaches for patients with NSCLC.

Circular RNAs (circRNAs) are recently a class of novel non-coding RNAs (ncRNAs) with circular configurations and have attracted much attention[5,6]. With the rapid development of RNA sequencing (RNA-Seq) technology, circRNAs have been confirmed to be dysregulated frequently in most cancers, and to play critical regulatory roles in the process of tumorigenesis and progression[7]. Several dysregulated circRNAs have been reported to be involved in NSCLC cell proliferation, invasion, migration, immune evasion, and chemotherapy resistance, for example circFGFR1[8], circNDUFB2[9], and circMET[10]. SOX4 is a member of the SOX (Sry-related high-mobility group box) transcription factor family[11]. The *SOX4* gene is frequently amplified and overexpressed in most malignancy tumors. Evidence increasingly demonstrates that SOX4 act as an oncogene in several cancer progression[12]. Bagati *et al.*[13] reported that increased expression of SOX4 induced immune evasion and promoted resistance to anti-PD-1 immunotherapy in triple-negative breast cancer.

Here, we reported a novel cancer immune evasion-related circRNA hsa_circ_0020714, which is significantly upregulated in NSCLC tissues compared with nontumor adjacent tissues. And forced expression of hsa_circ_0020714 is associated with immune evasion of NSCLC and predicts a poor prognosis. Functionally, knockdown and overexpression experiments indicated that hsa_circ_0020714 regulates the sensitivity of NSCLC to anti-PD-1 immunotherapy. Moreover, hsa_circ_0020714 acts as a sponge for miR-30a-5p to upregulate SOX4 expression and consequently induces NSCLC immune evasion. This study reveals an innovative new insight into the underlying molecular mechanism of NSCLC immune evasion and sheds light on hsa_circ_0020714 as a promising sensitivity prediction biomarker and a potential therapeutic target for NSCLC immunotherapy.

METHODS

Cell culture

Human NSCLC cell lines (A549, HCC827, NCI-H1299, NCI-H1650, NCI-H226, and NCI-H460) and HEK-293T cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

These cells were routinely cultured in DMEM medium (HyClone, Logan City, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, USA) and 1% penicillin/streptomycin (100 IU/mL) at 37 °C in a humidified incubator with 5% CO_2.

Patients and tissues

We obtained NSCLC tissues and matched adjacent non-tumoral tissues from patients who underwent surgery at the Zhongshan Hospital of Fudan University and Second Affiliated Hospital of Nanchang University. The tissues from NSCLC patients were counterstained with hematoxylin and eosin and were confirmed independently by two independent pathologists. All patients or their guardians gave written informed consent for the use of their samples before collection. This study received approval from the Ethical Review Committee of the Zhongshan Hospital of Fudan University.

Total RNA extraction and quantitative real-time polymerase chain reaction detection

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis were performed according to our previous studies[8,14,15]. In brief, total RNA from NSCLC tissues, matched adjacent non-tumor tissues, and NSCLC cell lines were extracted using TRIzol (Invitrogen, USA) reagent and then reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Japan). Finally, qRT-PCR was carried out with SYBR Green Real-time PCR Master Mix (Yeasen, Shanghai, China) following the manufacturer's instructions.

Mice xenograft anti-PD-1 therapy study, western blot, circRNA immunoprecipitation, RNA pull-down, and dual luciferase reporter assays

Mice xenograft anti-PD-1 therapy study, western blot, circRNA immunoprecipitation (circRIP), RNA pull-down, and dual luciferase reporter assays were evaluated with SPSS software (19.0, SPSS, Inc., Chicago, IL) as described in our previously studies[8,10,14]. Experimentation on *C57BL/6* mice were approved by the Animal Experimentation Ethics Committee of Zhongshan Hospital, Fudan University. And the antibody used in western blot and circRIP assays were listed as follows: Tubulin (Abcam, ab52623), AGO2 (Abcam, ab186733), and SOX4 (Abcam, ab70598).

Transfection of lentiviral vectors, plasmids, and miRNA mimics

Transfection of lentiviral vectors, plasmids, and miRNA mimics were evaluated with SPSS software (19.0, SPSS, Inc., Chicago, IL) as described in our previously studies[10,14]. In brief, Hsa_circ_0020714 overexpression lentiviral vectors, miR-30a-5p mimics, or pLG3 plasmid containing the sequence of wild type/mutant hsa_circ_0020714/ SOX4 mRNA 3′-UTR were constructed (Shanghai Genomeditech Co. Ltd., Shanghai, China). Transfectant cells were characterized by qRT-PCR or western blotting.

Statistical analysis

Statistical analysis was conducted using the SPSS software (19.0, SPSS, Inc., Chicago, IL) as described in our previously studies[10,14]. $P < 0.05$ was considered statistically significant.

RESULTS

Hsa_circ_0020714 is highly expressed in NSCLC tissues and is correlated with poor prognosis

Previous studies have indicated that forced CD151 expression in NSCLC was correlated to poor prognosis[16]. Importantly, CD151-derived circRNA circ_0020710 was significantly overexpressed in melanoma tissues and its upregulated expression induced melanoma immune evasion[17]. Therefore, we speculated that the dysregulation of CD151-derevied circRNAs may act as oncogenes in NSCLC cells. To explore CD151-derived circRNA expression in NSCLC tissues and matched adjacent nontumor tissues, we examined the CD151-derived circRNAs expression in four pairs of NSCLC tissues and matched adjacent

nontumor lung tissues by using qRT-PCR. The results indicated that hsa_circ_0020714 was upregulated in the NSCLC tissues [Figure 1A]. Next, we examined hsa_circ_0020714 expression in the NSCLC tissues and matched adjacent nontumor tissues and found that hsa_circ_0020714 was significantly increased in the NSCLC tissues (tumor/non-tumor ≥ 2) (74/120) [Figure 1B]. Furthermore, our results demonstrated that patients with upregulated hsa_circ_0020714 expression had a worse prognosis than those with lower levels of hsa_circ_0020714 [Figure 1C and D].

Higher levels of hsa_circ_0020714 expression is correlated with resistance to anti-PD-1 therapy in NSCLC patients

Increasingly, studies have reported the dysregulation of circRNAs execute a critical role in cancer immune evasion[8,18]. Therefore, we explored whether forced hsa_circ_0020714 expression can decrease the curative effect of anti-PD-1 therapy (Keytruda). Then, we analyzed retrospective data from 42 NSCLC patients (29 cases of lung adenocarcinoma and 13 cases of lung squamous cell carcinoma) with relapse or distant metastasis receiving anti-PD-1 immunotherapy. After six therapy cycles, the efficacy was examined using enhanced computerized tomography and assessed based on iRECIST criterion. For 29 cases of lung adenocarcinoma, the results demonstrated that there were 4 patients with partial response, 14 patients with stable disease, and 11 patients with progressive disease. For 13 cases of lung squamous cell carcinoma, there were 1 patient with partial response, 5 patients with stable disease, and 7 patients with progressive disease. Next, hsa_circ_0020714 expression levels were evaluated via qRT-PCR, and the results demonstrated that the expression levels of hsa_circ_0020714 were higher in the progressive disease group compared with stable disease and partial response groups in both adenocarcinoma and squamous cell carcinoma panels [Figure 2A and B]. These results showed that hsa_circ_0020714 likely participates in the immune evasion and resistance to anti-PD-1 immunotherapy of NSCLC.

Hsa_circ_0020714 upregulates SOX4 expression sponging miR-30a-5p

Increasingly, studies have verified that circRNAs act as miRNA sponges. We therefore analyzed whether hsa_circ_0020714 has the ability to induce immune evasion by sponging certain miRNAs. Through StarBase v3.0 analysis, we found that hsa_circ_0020714 and SOX4 were predicated to be possible targets of miR-30a-5p [Figure 3A]. Importantly, it has been reported that forced SOX4 expression promoted immune evasion and resistance to anti-PD-1 immunotherapy in triple-negative breast cancer[13]. Based on these results, we speculated that hsa_circ_0020714 promoted NSCLC immune evasion possibly via sponging miR-30a-5p to upregulate SOX4. To further determine the function of hsa_circ_0020714 in immune evasion , we detected hsa_circ_0020714 expression in seven human NSCLC cell lines [Figure 3B]. Next, RIP experiment with argonaute 2 (AGO2) antibody in A549 cells was performed. Our results indicated that hsa_circ_0020714, SOX4 mRNA, and miR-30a-5p, but not circANRIL (a circular RNA that confirmed does not bind to AGO2)[19], were significantly enriched [Figure 3C]. Furthermore, we performed luciferase assays using miR-30a-5p mimics cotransfected with luciferase reporters (which contained a wild-type or miR-30a-5p-target mutant hsa_circ_0020714 sequence/SOX4 mRNA 3′-UTR) into HEK-293 T cells. Compared with the negative control mimics, miR-30a-5p significantly inhibited the luciferase reporter activity in the cells with the wild-type hsa_circ_0020714/SOX4 mRNA 3′-UTR sequence, but not affect the activity in the cells with the miR-30a-5p-target mutant circFGFR1/SOX4 mRNA 3′-UTR sequence [Figure 3D]. To determine whether hsa_circ_0020714 upregulates SOX4 expression via the miR-30a-5p, we established hsa_circ_0020714 or miR-30a-5p target sequence of mutant hsa_circ_0020714 NCI-H460 overexpression cell lines through lentiviral vectors [Figure 3E]. The mRNA and protein levels of SOX4 were significantly increased in NCI-H460 cells following transfection with hsa_circ_0020714, compared with the mock group and mutant hsa_circ_0020714 group [Figure 3F and G].

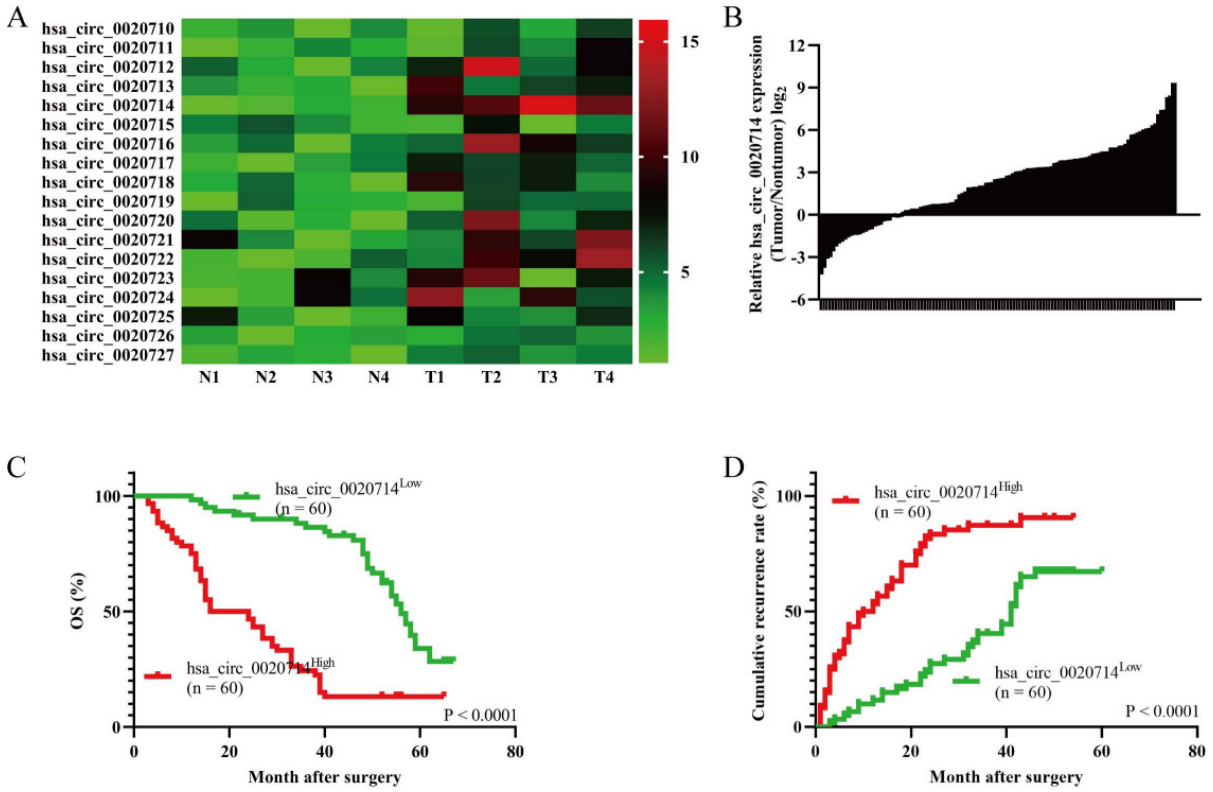

Figure 1. Forced hsa_circ_0020714 expression in the non-small cell lung cancer (NSCLC) tissues correlated with poor prognosis. (A) The heatmap shows *CD151* gene-derived circRNAs in pairs of NSCLC tissues and adjacent nontumor tissues using qRT-PCR. (B) The differential expression of hsa_circ_0020714 in the NSCLC tissues and matched adjacent nontumor tissues of 120 patients, as indicated. (C, D) Prognostic analysis of hsa_circ_0020714 expression in 120 NSCLC patients.

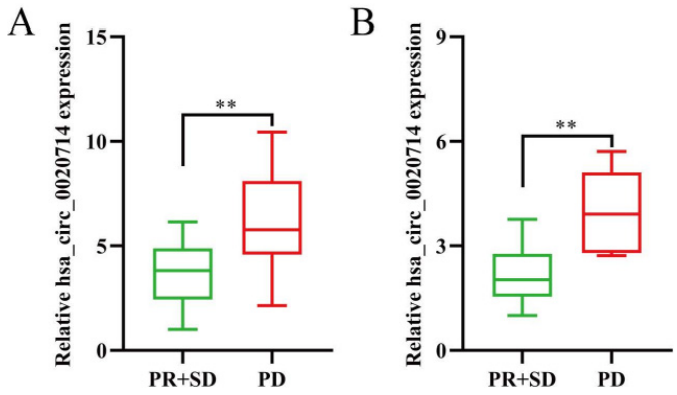

Figure 2. Hsa_circ_0020714 may act as a biomarker for resistance to anti-PD-1 immunotherapy in non-small cell lung cancer (NSCLC) patients. (A) Lung adenocarcinoma. (B) Lung squamous cell carcinoma. $^{**}P < 0.01$.

To further verify whether hsa_circ_0020714 upregulates the SOX4 expression by sponging miR-30a-5p, we detected the expression of miR-30a-5p and SOX4 mRNA in above 120 NSCLC patient tumor tissues. The results demonstrated that there was a negative correlation between miR-30a-5p and hsa_circ_0020714/SOX4 mRNA in the NSCLC tissues. Inversely, positive correlations between hsa_circ_0020714 and SOX4 mRNA was observed in the NSCLC tissues [Figure 4A-C]. Moreover, our results demonstrated that patients with upregulated SOX4 mRNA expression had a worse prognosis than

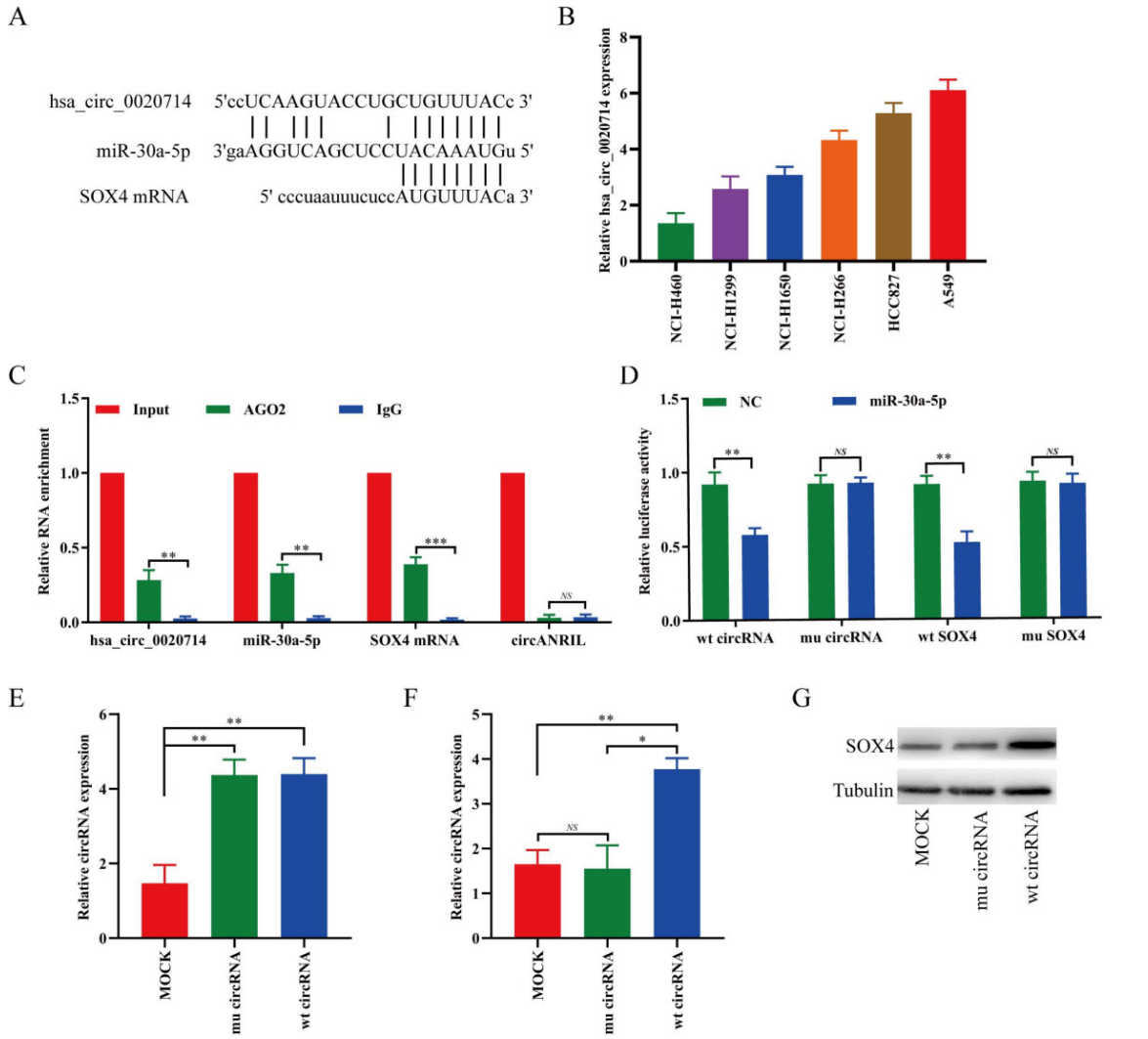

Figure 3. Hsa_circ_0020714 upregulates SOX4 expression via sponging miR-30a-5p in NSCLC cells. (A) Putative miR-30a-5p binding sites to hsa_circ_0020714 and SOX4 were predicated via StarBase v3.0. (B) Hsa_circ_0020714 expression was detected in six NSCLC cell lines using qRT-PCR. (C) CircRIP analysis were carried out using an AGO2 antibody in A549 cells extract. (D) The luciferase activity of wild type (wt) or mutant (mu) pLG3-hsa_circ_0020714/SOX4 mRNA 3′-UTR in the HEK-293T cells after cotransfected with miR-30a-5p. (E) Hsa_circ_0020714 expression was modified in the NCI-H460 cells by lentivirus-mediated cDNA transfection. (F, G) SOX4 mRNA and protein expression levels in wild type (wt) or mutant (mu) hsa_circ_0020714-overexpressing NSCLC cells. ***P < 0.001, **P < 0.01, *P < 0.05. NS: Not significant.

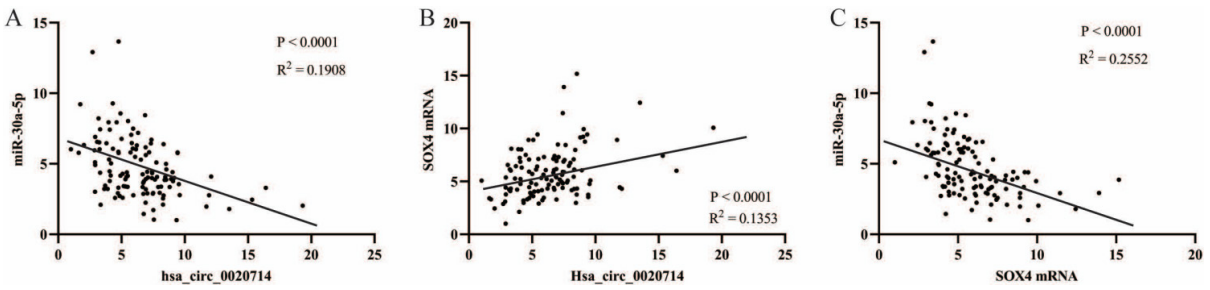

Figure 4. The correlation between hsa_circ_0020714, miR-30-5p, and SOX4 mRNA was analyzed in NSCLC tissues. (A) The correlation between hsa_circ_0020714 and miR-30-5p was analyzed in NSCLC tissues; (B) The correlation between hsa_circ_0020714 and SOX4 mRNA was analyzed in NSCLC tissues; (C) The correlation between miR-30-5p and SOX4 mRNA was analyzed in NSCLC tissues.

those with lower levels of SOX4 mRNA, and patients with increased miR-30a-5p expression had a better prognosis than those with lower levels of miR-30a-5p [Figure 5A-D].

Hsa_circ_0020714-induced resistance to anti-PD-1 therapy in a SOX4-depedent manner

Interestingly, humans and mice have the same miR-30a-5p sequence, and mouse SOX4 mRNA 3′-UTR also has a predicted target sequence of miR-30a-5p [Figure 6A]. Then, we performed a luciferase assay using miR-30a-5p mimics that were cotransfected with luciferase reporters (containing wild-type or the mutant miR-30a-5p target sequence of mouse Sox4 mRNA 3′-UTR) into HEK-293 T cells. Compared with the negative control mimics, miR-30a-5p significantly inhibited the luciferase reporter activity in the cells with the wild-type mouse Sox4 mRNA 3′-UTR, but not the cells with the mutant mouse SOX4 mRNA 3′-UTR [Figure 6B]. In addition, our results indicated that upregulated hsa_circ_0020714 significantly promoted SOX4 expression in mouse lung cancer LLC cell line [Figure 6C]. To further determine the biological function of hsa_circ_0020714 on anti-PD-1 therapy resistance, we detected the anti-tumor effects of the PD-1 antibody in LLC-hsa_circ_0020714 xenograft *C57BL/6* mice. Compared to that of the miR-30a-5p target sequence of mutant hsa_circ_0020714 group, the tumor growth increased in the LLC-hsa_circ_0020714 cells group which exhibited resistance to anti-PD-1 therapy and had a shorter survival time [Figure 6D-F].

DISCUSSION

With the rapid development in the fields of bioinformatics and high-throughput sequencing techniques, the researchers have gradually deepened their understanding of circRNAs. Increasingly studies have verified that dysregulated circRNAs expression play critical roles in NSCLC immune evasion and resistance to anti-PD-1 immunotherapy. For example, our previous study demonstrated that forced circFGFR1 promotes resistance to anti-PD-1 treatment in NSCLC immunotherapy via sponging miR-381-3p to upregulate the expression of C-X-C motif chemokine receptor 4[8]. In another study, our results indicated that circMET overexpression enhanced NSCLC immune evasion via acting as a miR-145-5p sponge to upregulate CXCL3 expression[10]. Here, for the first time we reported that hsa_circ_0020714 plays a critical biological function in NSCLC immune evasion. We determined that hsa_circ_0020714 was upregulated in NSCLC tissues compared with paired adjacent nontumor tissues, and forced hsa_circ_0020714 expression was associated with a poor prognosis and resistance to anti-PD-1 immunotherapy in NSCLC patients. Mechanistically, hsa_circ_0020714 functions as an endogenous miR-30a-5p sponge to enhance SOX4 expression, subsequently inducing resistance to anti-PD-1 immunotherapy in NSCLC patients.

CircRNAs belong to a novel class of important ncRNAs involved in regulating various pathological and physiological processes. In recent years, they have attracted numerous research attention. Mounting evidence shows that dysregulated expression of circRNAs play a critical role in NSCLC progression and immune evasion[8,10]. In previous study, we proved that circMET overexpression can promote cancer cell proliferation, metastasis, and immune evasion in NSCLC[10]. In addition, we found that forced circFGFR1 expression promoted NSCLC progression and resistance to anti-PD-1 by sponging miR-381-3p[8]. In this study, we found that hsa_circ_0020714 was a critical oncogene that participates in immune evasion of NSCLC.

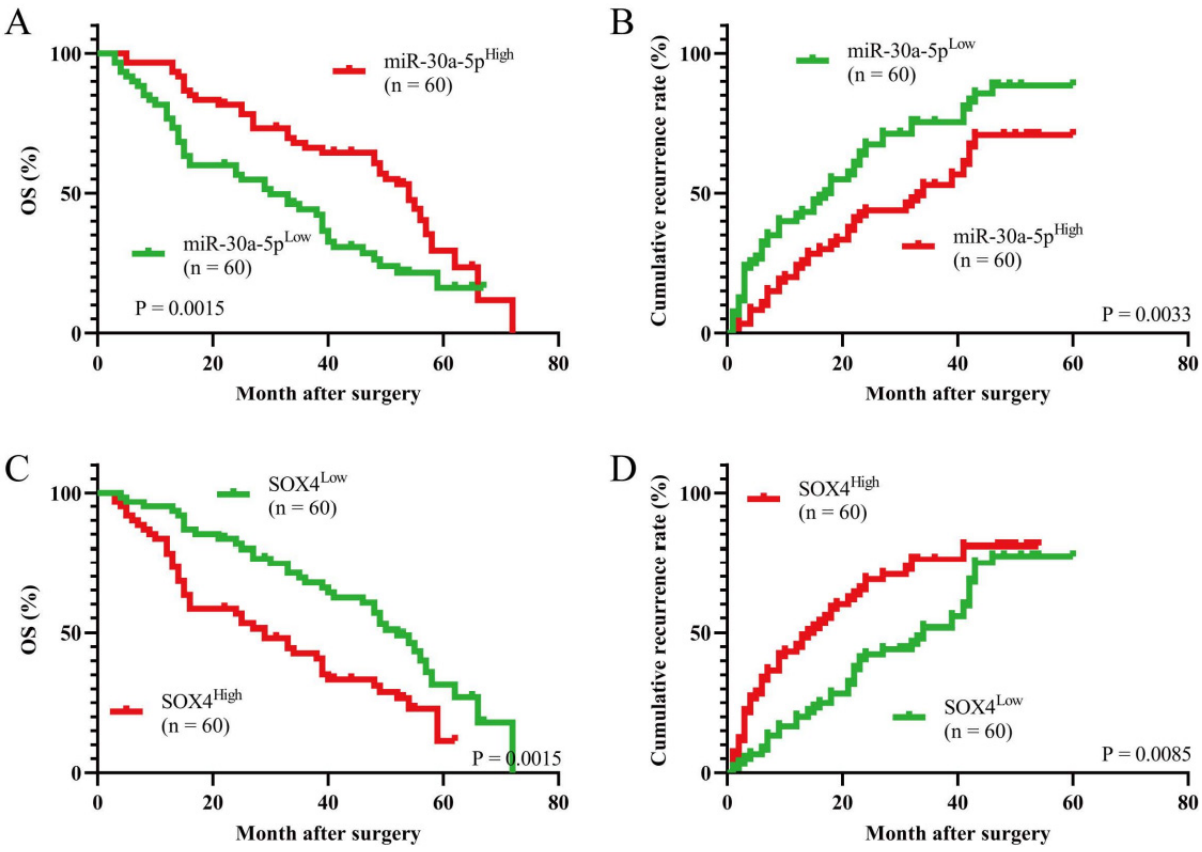

Figure 5. Prognostic analysis of miR-30-5p, and SOX4 mRNA expression in 120 non-small cell lung cancer (NSCLC) patients. (A and B) Prognostic analysis of miR-30-5p expression in 120 NSCLC patients; (C and D) Prognostic analysis of SOX4 mRNA expression in 120 NSCLC patients.

SOX4 is a member of the SOX family of transcription factors. Upregulated SOX4 expression participates in cancer progression, including proliferation, invasion, and migration[11]. In recent years, increasingly evidence has confirmed that the expression of SOX4 was regulated by a large class of ncRNA[20-22]. Our study identified SOX4 as a downstream important molecular of hsa_circ_0020714/miR-30a-5p axis in NSCLC cells. Recently, Bagati *et al.*[13] found that forced SOX4 expression inhibits T cell-mediated anti-tumor immunity in triple-negative breast cancer. Furthermore, PD-1 blockade was verified to efficiently promote proliferation and expansion of $CD8^+$ T cells in cancer model[23]. Over the past years, cancer immunotherapies have been demonstrated effectively therapeutic effect for some patients by blocking immune checkpoint receptors on T cells, restoring T cell-mediated cytotoxicity and recruiting more T cells into the tumor environment[24]. Here, our results also indicated that hsa_circ_0020714 could sponge miR-30a-5p to upregulate SOX4 expression in the NSCLC cells, thereby promoting NSCLC immune evasion and resistance to anti-PD-1 immunotherapy.

Taken together, our results demonstrate that hsa_circ_0020714 expression is upregulated in NSCLC tissues compared with the paired adjacent nontumor tissues. Mechanistically, hsa_circ_0020714 induces the immune evasion of NSCLC cells via sponging miR-30a-5p to upregulate SOX4 expression, which has been confirmed acted as a oncogene in several malignant tumors, including NSCLC. Therefore, blocking the hsa_circ_0020714/miR-30a-5p/SOX4-related pathway may effectively reverse resistance to anti-PD-1-based immunotherapy in NSCLC.

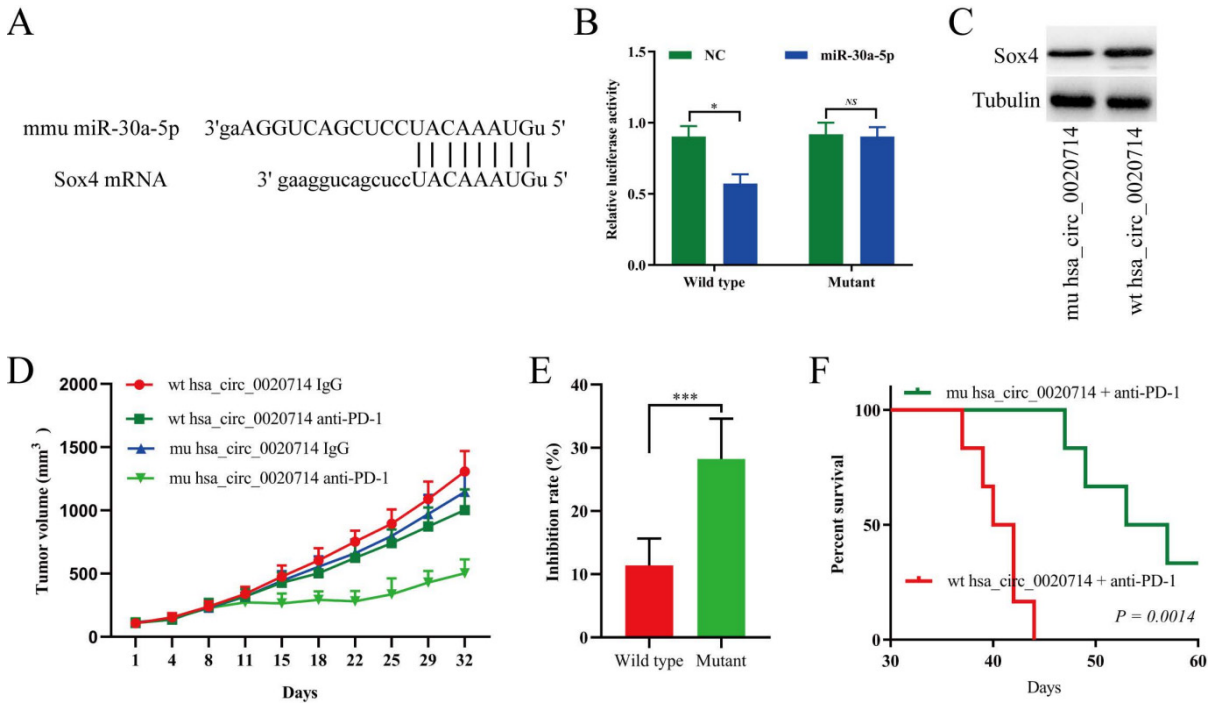

Figure 6. Hsa_circ_0020714 promotes non-small cell lung cancer (NSCLC) resistance to resistance to anti-PD-1 immunotherapy in NSCLC patients. (A) Putative miR-30a-5p binding sites to mouse Sox4 were predicated via StarBase v3.0. (B) The luciferase activity of wild type (wt) or mutant (mu) pLG3-Sox4 mRNA 3′-UTR in the HEK-293T cells after cotransfected with miR-30a-5p. (C) Sox4 expression in the wild type or mutant hsa_circ_0020714-overexpressing cells was detected by western blot. (D) LLC cells with mutant or wild type hsa_circ_0020714-overexpressing were subcutaneously injected into 4-week-old *C57BL/6* mice. When tumors had reached a mean volume of 100 mm^3, the mice were treated with an IgG or PD-1 antibody. The data are presented as the mean tumor volume (n = 6). (E) The data are expressed as the percentage of tumor growth inhibition (the data are presented as the mean ± SD). (F) The survival curves of the mouse lung xenograft tumors formed by mutant or wild type hsa_circ_0020714-overexpressing cells and treated with a mouse antibody against mouse PD-1. ***P < 0.001, *P < 0.05. NS: Not significant.

DECLARATIONS

Authors' contributions

Conceived and designed the experiments: Wu J, Zhu MX, Li KS
Performed the experiments: Wu J, Zhu MX, Li KS, Zhang PF
Analyzed the data: Zhang PF, Peng L
Wrote the paper: Zhang PF, Peng L
All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed in this study are included in this article.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee. Experimentation on C57BL/6 mice were approved by the Animal Experimentation Ethics Committee of Zhongshan Hospital, Fudan University. All patients or their guardians gave written informed consent for the use of their samples before collection.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Editorial

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Cancer drug resistance in multiple myeloma

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INTRODUCTION

Multiple myeloma (MM) is the second most common hematologic malignancy after non-Hodgkin's lymphoma. Intrinsic and acquired drug resistance of cancer cells to standard drugs is a major obstacle for a more successful survival outcome of MM patients treated on contemporary clinical protocols. The primary purpose of this special issue on "Drug Targets and Resistance Mechanisms in Multiple Myeloma" was to collect new and transformative information regarding new insights about the mechanisms of drug resistance in MM and the role of the tumor microenvironment in treatment failures.

MAIN TEXT

Overcoming inherent and acquired drug resistance of MM cells, especially in the context of the immunosuppressive tumor microenvironment (TME), remains a major challenge to effective therapy of high-risk or relapsed/refractory (R/R) MM[1-15]. Amplified expression of drug transporters P-glycoprotein (P-gp/ABCB1) and multidrug-resistance-associated protein 1 (MRP1/ABCC1) have been implicated in the resistance of MM cells to drugs that are known substrates for these proteins, such as melphalan, dexamethasone, and anthracyclines [Table 1][16-18]. Some studies have indicated that abundant expression levels of the lung resistance protein (LRP), another drug transporter protein, may also confer resistance to chemotherapy drugs and proteasome inhibitors (PIs) in MM[16-18]. It remains to be seen if a potent and safe inhibitor of these drug transporters can be identified and clinically leveraged to overcome drug resistance in

Table 1. Possible Mechanisms of Cancer Drug Resistance in Multiple Myeloma

Therapeutics	Resistance Mechanism
IMiD	Mutations of proteins in CRBN-IK axis associated with deficient expression
PI	Impaired binding to PSMB5 due to mutations; MIF overexpression; LRP abundance; epigenetic reprogramming; DNA hypermethylation in an active intronic CRBN enhancer
Chemo	Amplified expression of P-gp/ABCB1 and MRP1/ABCC1
MoAb	Low expression level of target antigen; upregulation of the complement inhibitors CD55 and CD59
DEX	GR mutations and deficiency; amplified expression of P-gp/ABCB1 and MRP1/ABCC1

DEX: Dexamethasone; IMiD: immuno-modulatory drug; PI: proteasome inhibitor; Chemo: chemotherapy drugs; MIF: migration inhibitory factor; LRP: lung resistance protein; P-gp: P-glycoprotein; MRP1: multidrug-resistance-associated protein 1.

MM. Several humoral and cellular components of the TME facilitate the immune evasion and subsequent expansion of drug-resistant MM clones, such as myeloid-derived suppressor cells (MDSCs), regulatory T-cells, and MM-derived cytokines including TGF-β, and IL-6, and IL-10[4]. MYC and hepatocyte growth factor/c-MET signaling networks have also been identified as potential contributors to cancer drug resistance in MM[15]. Patients with triple-class-refractory MM whose cells exhibit triple-class resistance to PIs, immunomodulatory drugs (IMiDs), and monoclonal antibodies (MoAb) have an OS of < 6 months emphasizing the urgency of this unmet medical need[19-20].

IMiDs such as thalidomide, lenalidomide, and pomalidomide trigger cereblon (CRBN)-mediated ubiquitination and degradation of important regulatory proteins that contribute to the expansion of drug-resistant clones, including the transcription factor Ikaros[18]. They also augment the proliferation and effector function of cytotoxic T-cells (CTLs) and natural killer (NK) cells while inhibiting immunosuppressive T-regs. Unfortunately, deficient expression of the target CRBN, as reported for MM cells with certain mutations of the *cereblon* gene or DNA hypermethylation in an active intronic CRBN enhancer, occurs in almost one-third of the patients with R/R MM and causes resistance to the IMiDs [Table 1][21,22]. CRBN-independent resistance to IMiDs has also been reported[1,4]. Ongoing research will explore if the clinical benefit of IMiDs could be augmented by using them in combination with CRBN E3-ligase modulators (e.g., iberdomide) to accelerate the degradation of Ikaros transcription factor proteins IKZF1/IKZF3[1,4].

In contemporary induction protocols for MM, IMiDs are often combined with PIs such as bortezomib and Carfilzomib[5,20]. PIs trigger apoptotic death of MM cells by contributing to an exaggerated unfolded protein response (UPR) pathway and ER stress[1,4]. They further impair the survival-promoting interactions between MM cells and stromal elements in the bone marrow microenvironment, and they promote the immunogenic death of damaged MM cells[1,4]. Impaired binding of PIs to the target proteasome β5 subunit (PSMB5) has been associated with PI resistance and can occur due to mutations of the encoding gene that cause conformational alterations at the binding region [Table 1][23,24]. MicroRNAs (miRNAs) play important roles in mRNA silencing and regulation of gene expression in MM cells[25]. CD47 antigen is overexpressed in MM, likely due to miR155 down-regulation, and its abundance was associated with a poor prognosis[26-28]. Recent studies have implicated CD47 in PI resistance of MM cells[28]. In view of the promising early clinical experience in patients with myeloid malignancies, evaluation of the anti-CD47 MoAb Magrolimab in R/R MM patients would also seem warranted[4]. Notably, CD47-targeting with synthetic micro-RNA miR-155 overcame bortezomib resistance and induced phagocytosis as well as apoptosis of MM cells by causing loss of CD47[28]. Likewise, another micro-RNA, miR-218, is decreased in MM and synthetic miR-218 may help overcome PI resistance[29]. In the future, nanoformulations of synthetic miR155 and miR-218 could be used for the resensitization of resistant MM cells to PI. Another emerging new strategy to overcome PI resistance

involves the targeting of the macrophage migration inhibitory factor, which has been shown to render MM cells resistant to PI-induced apoptosis[30]. Clinical biomarker studies have demonstrated that the high-level expression of this target is associated with poor prognosis and survival in MM[30].

BCL-2 is a predominant anti-apoptotic protein in B-lineage lymphoid malignancies, including MM. Venetoclax is a BCL-2 homology 3 (BH3)-mimetic that disrupts the association of the proapoptotic BH3-only proteins such as BIM and BID with BCL-2[31]. BCL-2 inhibition by Venetoclax could theoretically damage chemotherapy-resistant MM cells by inhibiting the amino acid metabolism and reducing oxidative phosphorylation like its effects on leukemic cell populations[1,32,33]. Venetoclax exhibited meaningful single-agent activity in R/R MM patients, especially those with a t(11;14) translocation[34]. A combination of Venetoclax plus Bortezomib and dexamethasone was more effective than placebo plus bortezomib and dexamethasone in patients with R/R MM, albeit with higher toxicity due to infections[35]. Besides BCL-2, MCL-1 is also an important survival-promoting anti-apoptotic protein for MM cells, and inhibitors of MCL-1 such as AMG-176 and MIK665 have been developed as potential anti-MM drugs that could be combined with other apoptosis-promoting anti-MM drug candidates including Venetoclax[36].

A recent prospective clinical study employed longitudinal single-cell RNA-sequencing (scRNA-seq) to study the mechanism and dynamics of drug resistance in MM[37]. An enzyme of the UPR pathway, peptidylprolyl isomerase A, was identified as a new molecular target demonstrating the clinical potential of this new strategy in identifying clinically relevant new therapeutic targets for overcoming cancer drug resistance in MM[37]. Another important strategy for further identification of new molecular targets contributing to cancer drug resistance in MM is deep measurable residual disease (MRD) profiling, which is based on the characterization of MRD clones using flow cytometry in combination with whole-exome sequencing[38].

Precision medicines as well as biotherapeutic agents, including therapeutic monoclonal antibodies such as the anti-CD38 MoAb Daratumumab and isatuximab, and the anti-signaling lymphocyte activation marker F7, antibody elotuzumab, antibody-drug conjugates, and bispecific antibodies (BiAb) have been developed to damage drug-resistant MM clones as well as alter the immunosuppressive bone marrow microenvironment with some very promising clinical data regarding their clinical impact potential[1,4,39]. T-cell redirecting BiAb and bispecific T-cell engagers (BiTES) targeting CD38, the orphan G protein-coupled receptor GPRC5D, and the B-cell maturation antigen (BCMA)/CD269 on MM cells and CD3 antigen on T-cells facilitate the CTL-mediated destruction of drug-resistant MM cells in cytolytic synapses[4,40]. They showed promising single-agent activity in early clinical trials of R/R MM patients, and risk mitigation strategies have been identified for their potentially serious side effects such as cytokine release syndrome and neurotoxicity[4,40]. BCMA-targeting cellular immunotherapy platforms using chimeric antigen receptor (CAR)-T cells or NK cells have also been developed with documented objective clinical responses in single-agent trials[1,4,6-9,40].

Another area of active clinical research to improve the outcome of cancer drug-resistant MM is related to efforts for overcoming the immunosuppressive TME[4,40]. BiAb and anti-CD38 MoAb are capable of dual targeting of both MM cells and immunosuppressive elements of the TME such as the MDSC, and are being explored as potential therapeutic platforms[40].

Each of the new modalities designed to mitigate or overcome cancer drug resistance in MM has faced inherent and acquired resistance mechanisms[21]. For example, soluble BCMA renders MM cells resistant to the cytolytic actions of BCMA-directed BiAb/BiTES and CAR-T cells by serving as a competing target for

these biotherapeutic platforms. It remains to be seen if this resistance can be overcome by using a γ-secretase inhibitor to reduce the γ-secretase mediated production of soluble BCMA. It will be important to develop multi-modality combination regimens to minimize the risk of escape by drug-resistant MM clones as well as the emergence of MM clones refractory to the new agents with promising activity[1,4,13,20,21,35,40-45]. The timely definition of optimal strategies for overcoming the cancer drug resistance in MM will require randomized adaptive clinical trials with multiple parallel cohorts, each evaluating a promising new treatment strategy.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

Availability of data and materials

Not applicable.

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Conflicts of interest

Author Fatih M. Uckun was employed by Ares Pharmaceuticals, and he was a consultant for Reven Pharmaceuticals. The author declares that this study did not receive any funding from any sponsor or commercial entity. No person other than the author was involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. The author declares no other competing interests.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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The complex relationship between multiple drug resistance and the tumor pH gradient: a review

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Abstract

Multiple drug resistance (MDR) is the tumor's way of escaping the cytotoxic effects of various unrelated chemotherapeutic drugs. It can be either innate or acquired. MDR represents the end of the therapeutic pathway, and it practically leaves no treatment alternatives. Reversing MDR is an unfulfilled goal, despite the important recent advances in cancer research. MDR, the main cause of death in cancer patients, is a multi-factorial development, and most of its known causes have been thoroughly discussed in the literature. However, there is one aspect that has not received adequate consideration - intracellular alkalosis - which is part of wider pH deregulation where the pH gradient is inverted, meaning that extracellular pH is decreased and intracellular pH increased. This situation interacts with MDR and with the proteins involved, such as P-gp, breast cancer resistance protein, and multidrug associated resistance protein 1. However, there are also situations in which these proteins play no role at all, and where pH takes the lead. This is the case in ion trapping. Reversing the pH gradient to normal can be an important contribution to managing MDR. The drugs to manipulate pH exist, and most of them are FDA approved and in clinical use for other purposes. Furthermore, they have low or no toxicity and are inexpensive compared with any chemotherapeutic treatment. Repurposing these drugs and combining them in a reasonable fashion is one of the points proposed in this paper, which discusses the relationship between cancer's peculiar pH and MDR.

Keywords: Multidrug resistance, pH gradient inversion, reversion of the pH gradient, P-gp, pH centered treatment.

INTRODUCTION

For more than 3000 years, trepanations (for unclear reasons) have been performed, since 2000 BC (and a breast cancer tumor was excised approximately at the same date); up to the 20th century, surgery was the only available treatment for solid tumors. During that time, there was nothing to be done for non-solid tumors. Things started to change in the middle of the last century.

Chemotherapy started in earnest in the 1940s, a decade in which two important advances achieved clinical status. In the first of these events, Louis Goodman and Alfred Gilman created the first alkylating agent as a derivative of the poisonous nitrogen mustard gas, a sibling of the sulfur mustard gas used in WWI[1]. The second event had Sydney Farber as the protagonist. He introduced the first anti-metabolite (aminopterin) for acute leukemia treatment in children[2]. Interestingly, the title of the first publication by Farber and his associates starts with "Temporary relief…", thus, incorporating from the first moment one of the main problems of chemotherapy, the limited duration of its benefits. In both cases, the first patients treated by Goodman and Gilman on the one hand and the children with acute leukemia treated by Farber *et al.*[2] on the other hand, the effects of chemotherapy were not long lived and repeat treatments were usually unsuccessful. Chemotherapy came up against its main adversary, resistance. To prolong the beneficial effects and at the same time reduce toxicity, multidrug chemotherapy protocols were introduced - successfully in many cases. Remissions lasted longer and toxicity was reduced. However, resistance and relapse still persisted at the end of the therapeutic path. Patients receiving chemotherapy can in many cases become resistant to previously effective drugs. Unfortunately, resistance is the proof of concept that cancer cells are the most adaptive cells in eukaryotes.

Resistance is the product of two different sources:

(a) The malignant cell itself; and

(b) The stroma and the vascular system.

The most important mechanism is the one in which the cancer cell develops the ability to prevent the drug from entering it or reduces the amount that can enter, simply expels the drug, or can inhibit apoptosis despite the treatment.

The stroma may contribute to resistance through its dense composition (e.g., pancreatic cancer with desmoplastic reaction) or its low vascular supply that decreases drug distribution in the tumor [Figure 1].

A distinction must be made between primary (or intrinsic) and acquired (or secondary) resistance. In the first case, cells are resistant to the drug before they first encounter it. In the latter, acquired resistance, the tumor develops resistance during the course of chemotherapy. This difference implies that, in the second case, tumors evolve from their initial responsive status towards an unresponsive one. The main evolution consists in upregulating the expression of some specific proteins of the ATP binding cassette family (ABC), namely P-glycoprotein (P-gp, also known as MDR1 or ABCB1), multidrug associated resistance protein 1 (MRP1 or ABCC1), and breast cancer resistance protein (BCRP or ABCG2).

The introduction of novel targeted therapies in the last twenty years was expected to dramatically change the resistance problem. That did not happen. Targeted treatments improved survival and progression-free periods in many different cancers - in some, a cure was even achieved - but resistance to treatment remained little changed in most cases.

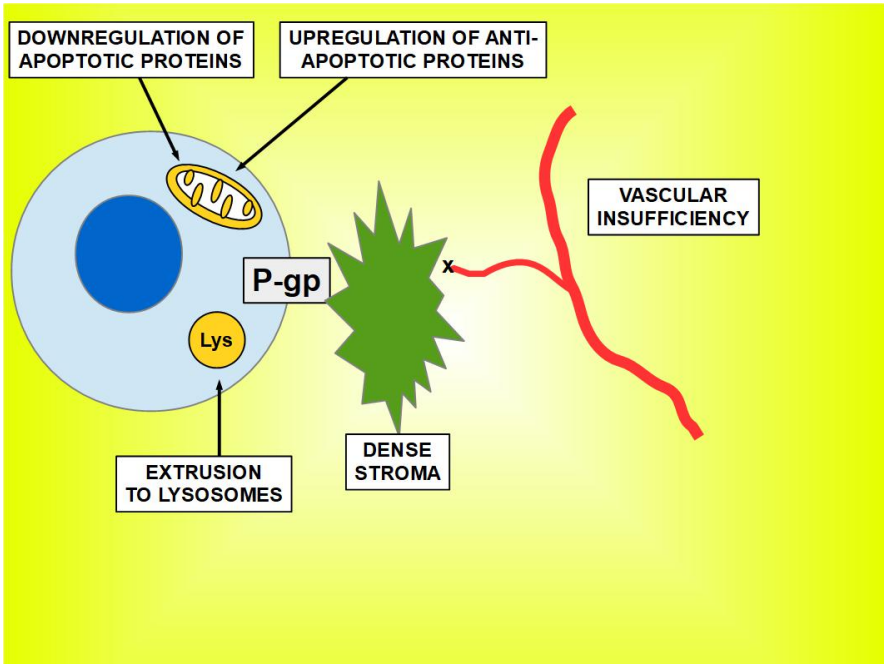

Figure 1. An overview of the main mechanisms of drug resistance. Drug extrusion is represented by P-gp (P-glycoprotein).

In 1958, Burchenal and Holmberg were the first to study the short-lived remission achieved with anti-metabolites in the treatment of leukemia at a cell level[3]. They expressed the prevailing concept at that moment - a random mutation induced by the drug - but they also established that there were many other biochemical mechanisms leading to resistance.

There is a special form of resistance consisting of invulnerability to many unrelated drugs that were never administered to the patient. This is known as MDR, a situation that severely limits the therapeutic options and consists of increased efflux of drugs. This mechanism was first proposed by Keld Danø, in 1973, as probably the main mechanism [4]. This drug extrusion hypothesis was followed some years later by the discovery of P-glycoprotein [5] and other MDR proteins such as multidrug- associated resistance protein 1 (MRP1) [6] and BCRP[7], proteins that were not known when Danø proposed his theory. This meant that the extrusion culprits were finally identified.

Multiple drug resistance

Resistance to a drug must be differentiated from multidrug resistance. In the first case, a mutation or clonal evolution can be the cause, supposing that the malignant cells are responsive to other chemotherapeutic drugs. It is the lack of response to many different drugs that signals towards MDR, especially if these drugs were never administered before. In this last case, the most probable cause is the increased expression of one or more of the MDR proteins. Therefore, it is cancer's resistance to many different and unrelated chemotherapeutic drugs that defines MDR, which may be intrinsic (primary) or acquired after chemotherapy. The lack of specificity is still one of the issues that is hard to explain. The causes of MDR are multiple, from poorly drug vulnerable stem cells to clonal evolution, stromal barriers, and mutations. It is not the aim of this paper to discuss MDR mechanisms but to focus exclusively on those related to pH alterations. When chemotherapy initially works, it is able to induce apoptosis in many of the malignant cells. However, tumors are very heterogeneous [8, 9] and not all malignant cells will be eliminated with the treatment. Those that survive repeated cycles of cytotoxic medication are either intrinsically resistant, or

they are located in inaccessible parts of the tumor, such as very hypoxic niches without blood supply or in the middle of dense connective tissue with high interstitial pressure impeding circulation [Figure 1]. The non-resistant cells are gradually killed, while the surviving resistant cells will thrive and progress and, ultimately, fully replace the "weaker" cells, thus heralding the relapse. This is a typically Darwinian evolution where chemotherapy is the selective force for the survival of the fittest.

For extensive reviews on MDR, read the works of Gillet*et al.*[10], Rascio *et al.*[11], Mansoori *et al.*[12] Jayaraj *et al.*[13], Ruan *et al.*[14], Aleksakhina *et al.*[15], and Vasan *et al.*[16].

This paper does not analyze MDR causes in depth, which is beyond the scope of the review, but rather focuses on the relationship between the tumor dysregulated pH and MDR and explores new therapeutic avenues in this regard.

pH deregulation in cancer

It has been known since the work of Otto Warburg [17] in the 1920s that tumors are acidic due to excessive production of lactic acid as a consequence of high glycolytic flux and downregulation of mitochondrial oxidative activity. Between the 1920s and the beginning of the 1970s, it was believed that there was no difference between intra- and extracellular pH. Thus, if the tumor was acidic, this concept included both sides of the cell membrane. Only in the late 1970s did it become evident that acidity was limited to the extracellular matrix, while in the intracellular milieu, pH was either unchanged or increased, compared with normal cells. The long time it took to discover the pH differences on the two sides of the cell membrane was due to the lack of adequate instruments that could accurately gauge intracellular pH [Table 1]. The awareness of different intra- and extracellular pH levels also led to the discovery of the channels, exchangers, transporters, and enzymes located on the membrane, which are in charge of maintaining this pH differential (gradient).

In Table 1, the direction of the arrow indicates the pH gradient, and we can easily see that in cancer, the gradient follows exactly the opposite direction compared with non-malignant counterparts. This is the inversion of the pH gradient, a process found in all tumors, which is fundamental for cancer cell survival and progression. This means that in cancer, extracellular pH becomes acidic and intracellular pH becomes more alkaline.

Extracellular acidity, known for a long time, and the more recently discovered intracellular alkalinity were merely considered as a consequence of cancer metabolism and, to a certain extent, innocent bystanders. This notion started to change in the mid-1980s, when researchers found more and more evidence showing that the inverted pH gradient represented an important advantage for growth, proliferation, migration, and invasion. Furthermore, in 2000, Reshkin *et al.*[18] showed that the first step in cellular transformation consisted in an increase of intracellular pH as a consequence of the enhanced activity of a specialized membrane channel, sodium bicarbonate exchanger 1 (NHE1). This exchanger, one of the main players in intracellular pH homeostasis, has the ability to export hydrogen ions (H^+, protons) while importing Na^+ from the matrix into the cells.

Reshkin *et al.*[18] went one step further; they inhibited NHE1 with a drug that has been in clinical use for more than fifty years, amiloride. Interestingly, NHE1 inhibition impeded malignization. This was clear proof of the importance of pH in cancerization.

Table 1. pH in different compartments in normal and cancer cells

Table 1	Normal cell	Cancer cell
Extracellular (EC) pH (pHe)	7.30-7.35	6.4-7.0
Intracellular (IC) pH (pHi)	7.2	7.25-7.50
pH gradient	EC→IC	IC→EC

Intracellular and extracellular pH are discussed separately below, even though they are part of the same process of pH deregulation. The membrane between both compartments is the tool that keeps this different pH alive. The membrane is not a simple and passive boundary, but rather it is an active player that maintains a different environment inside and outside the cell. This is in part achieved by channels, exchangers, transporters, and enzymes spanning through it.

Extracellular and intracellular pH (pHe and pHi)

Normal extracellular pH, which is very close to the blood pH, decreases by around 8–10% in cancer tissues. It goes from 7.35 (normal tissues) to roughly 6.8 (malignant tissues). In some cases, it becomes even more acid. This is the result of different events:

(a) Increased CO_2 production that produces carbonic acid on the cell surface and immediately ionizes, generating a bicarbonate ion that is reintroduced into the cell and a proton that remains in the extracellular matrix [Figure 2]. Two proteins located in the cell membrane participate in this process, namely membrane carbonic anhydrases (isoforms CAIX and CAXII) and sodium-bicarbonate cotransporter (NBC). CAIX is usually overexpressed in many hypoxic tumors[19-22]. CAIX is so closely associated with hypoxia that many authors consider its overexpression as a hypoxia marker[23-29].

CO_2 abandons the cell simply by diffusion. When it reaches the cell surface, there is a tandem activity on CO_2 carried out first by membrane carbonic anhydrase IX or XII and then by NBC [Figure 2].

Since Warburg's work and until 1999, lactic acid was considered the main culprit of extracellular acidosis in cancer. Seminal research by Newell *et al.*[30] showed that eliminating lactic acid production in malignant cells only minimally modified extracellular acidosis; thus, lactate is not the main and sole origin of a low pHe. It seems that CO_2 production is equally important in pHe descent. Malignant cells produce large amounts of CO_2 through the very active pentose phosphate pathway and fatty acid beta-oxidation.

(b) Increased lactic acid production is extruded from the cell by specialized membrane transporters such as monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4). Figure 3 shows the origin of lactic acid from the glucose metabolism, which is strongly deviated towards the glycolytic pathway instead of the oxidative pathway of the Krebs cycle. MCTs can carry monocarboxylates in general; they are not exclusively dedicated to lactate transport. In cancer, these transporters are overexpressed on the cell membrane[35-38]. Hao *et al.*[39] found that there was an association of CD44, CD147, MDR1, and MCTs expressions with prostate cancer progression and drug resistance.

(c)There is the extrusion of intracellular protons through NHE1, vacuolar ATPase proton pumps.

(d)There is the extrusion of protons through endosomes that become exosomes or simply release their acidic cargo into the extracellular matrix. Endosomes can follow two pathways: (a) transformation into lysosomes; and (b) becoming carriers of compounds that will be extruded from the cell, such as protons,

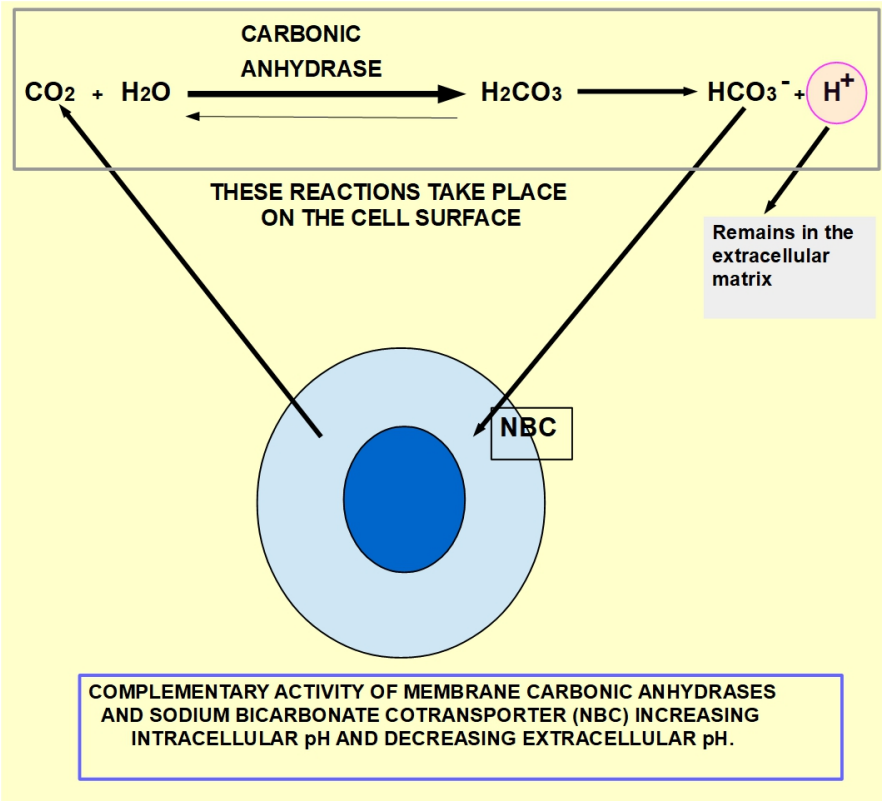

Figure 2. CO_2 of metabolic origin diffuses to the cell surface where it is hydrated to carbonic acid, which spontaneously ionizes to form a proton and a bicarbonate molecule. While the bicarbonate is reintroduced into the cell by NBC, the proton remains in the extracellular matrix, contributing to its acidification. NBC: Sodium-bicarbonate cotransporter.

proteolytic enzymes, etc.

(e)Debris of stromal and tumoral cell death are produced, whether by hypoxia, invasion, chemotherapy, or radiotherapy. Necrotic cells are able to release acidic intracellular compounds, while apoptotic cells are engulfed by macrophages and there is no intracellular content released into the matrix.

Reducing extracellular pH simultaneously increases intracellular pH because the mechanism is essentially based on protons released from inside the cell and into the matrix.

Matrix acidification has advantages for malignant cells because acidity:

• Decreases and inhibits immunological attacks on the tumor;

• Activates proteolytic enzymes needed for invasion; and

• Stimulates migration at the invadopodium level,

As mentioned above, intracellular pH increases through the loss of protons towards the matrix and the import of bicarbonate. This situation is also advantageous for the malignant cell because it:

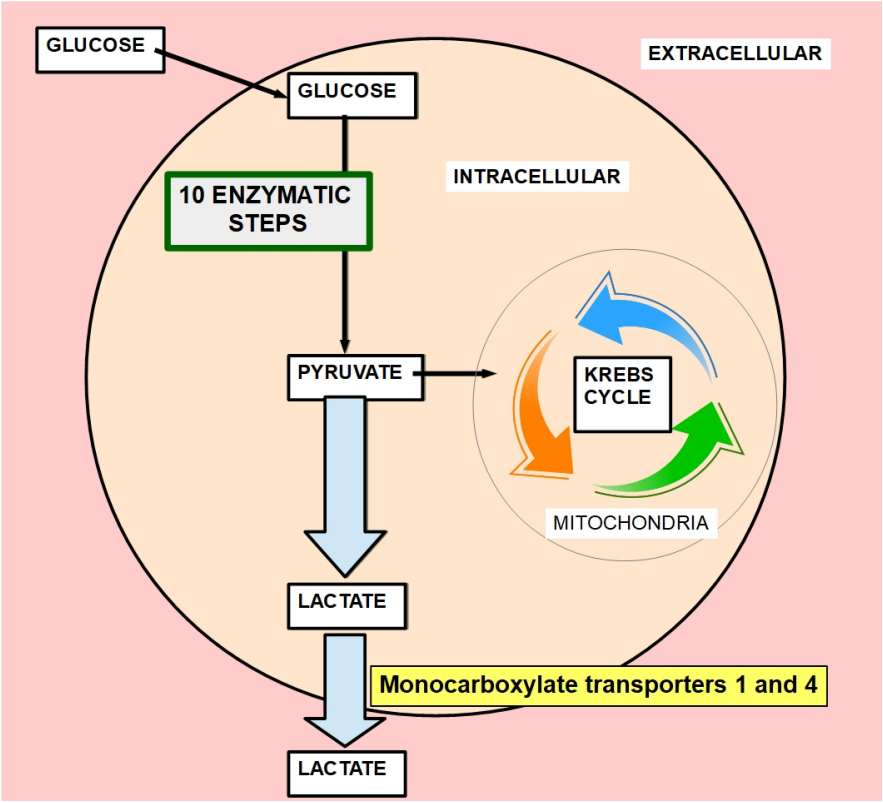

Figure 3. Origin of lactate production. Normal cells do not use the glycolytic pathway beyond pyruvate that goes into the Krebs cycle associated with coenzyme A and converted into acetyl-CoA. Malignant cells follow the glycolytic pathway ending in lactate that is extruded by the activity of monocarboxylate transporters. Lactate cannot stay inside the cell because it would decrease intracellular pH to life-threatening levels, thus it must be swiftly exported. This soft-spot of cancer metabolism transforms MCTs into valid targets[31-34].

• Increases the activity of glycolytic enzymes, thus enhancing glycolytic flux that allows a higher biosynthetic activity;

• Increases proliferation; and

• Decreases the possibility of apoptosis.

RELATIONSHIP BETWEEN DEREGULATED PH AND MDR

The relationship between pH and MDR has major participation in the resistance process, as is discussed below. We do not have the evidence to maintain that MDR would not be possible without deregulated pH; however, there are many findings that hint towards this hypothesis. At this point, we are convinced that restoring the normal pH gradient should be an integral part of MDR targeting.

The relationship between extracellular pH and MDR has been extensively studied and is represented by a phenomenon called ion trapping.

Ion trapping

Weakly basic chemotherapeutic drugs, such as doxorubicin, cannot enter the hydrophobic cell membrane because they undergo ionization in the acidic tumor extracellular environment. The lipid bilayer cell membrane is semipermeable, meaning that, while it allows fat-soluble non-ionized moieties to enter, it is

poorly permeable to ionized water-soluble molecules. Anthracyclines and vinca alkaloids are weak basic drugs that are ionized by the microenvironmental acidity, thus substantially reducing their access to the cell[40-42].

There are many reports that serve as proof of concept regarding the relation between ion trapping and extracellular acidity:

• Reducing extracellular acidity with sodium bicarbonate, mitoxantrome cellular penetration was increase[43].

• Gu *et al.*[44] developed a fluorescent probe based on dihydroberberine that showed that ionized berberine had a substantially lower cell penetration than the non-ionized form.

• Proton pump inhibitors, which increase extracellular pH, decrease drug resistance[45].

Endosomal ion trapping

Martinez-Zaguilán *et al.*[46] described the trapping of chemotherapeutic drugs in cellular endosomes. A new membrane is rapidly formed around the drug molecules, and these endosomes have a high V/ATPase proton activity, meaning that their interior is highly acidic. According to our criteria, drug release from the endosomes is inhibited by a mechanism similar to ion trapping. That is, the acidic interior ionizes weak basic drugs, impeding their transit through the membrane.

Extracellular acidity induces P-gp expression

This mechanism is independent of ion trapping and consists of the increased P-gp expression when extracellular pH becomes acidic[47-50]. The mechanism that leads from extracellular acidity to increased P-gp proteins has not been fully clarified as yet.

Intracellular alkalinity and its effects on MDR

The intracellular milieu and its sub-compartments, such as mitochondria, Golgi, endoplasmic reticulum, and endosomes, including autophagosomes and lysosomes, all have different pH, which is set according to the needs of the metabolic processes taking place in them:

• Cytoplasm is the site of glycolysis and fatty acid synthesis.

• Mitochondria are the site of the Krebs cycle, the electron transport chain, and lipid beta-oxidation.

• Golgi and endoplasmic reticulum mature proteins and secretions.

• Autophagosomes recycle organelles and other nutritional agents.

• Lysosomes and endosomes are very acid, degrade biological products, and intervene in the maturation of proteolytic enzymes.

Each of these activities requires a different optimum pH, and the cell's homeostatic machinery maintains these different pHs (gradients) through membranes, e.g., cytoplasmic pH around 7.2, mitochondrial pH around 8, and lysosomal pH below 5.5.

Cell proliferation requires an alkaline cytoplasm, slightly above that of the resting cell.

In an extensive phylogenetic review, Busa and Nucitelli[51] showed that almost all species increase their intracellular pH before replication. There were also some minor exceptions. This led the authors to consider intracellular pH as a signaling mechanism. High intracellular pH does not seem to be an indispensable mechanism but rather a facilitator for cell replication.

Drug-resistant cells were found to over-express or increase the activity of some of the membrane proteins discussed above[52]:

• A subunit of a vacuolar H+-ATPase proton pump[53]; and

• NHE1 (sodium hydrogen exchanger 1)[54-56].

Proton pumps and NHE1 [see Figure 4] are directly related to the pH gradient inversion: increasing intracellular alkalinization and decreasing extracellular pH. P-gp has a direct connection with intracellular and extracellular pH because:

• Intracellular acidification downregulates P-gp[57]; and

• Extracellular acidity increases P-gp expression by up to 5-10-fold *in vitro* [58].

This particular P-gp/pH relationship may explain some cases of intrinsic resistance, where there was no previous contact with any chemotherapeutic drug.

Apoptosis requires intracellular acidosis

The main objective of chemotherapy consists in inducing the programmed death (apoptosis) of malignant cells[59, 60]. If this objective is not achieved, the tumor is resistant[61]. Tumors have the ability to suppress apoptosis to a certain extent[62]. There are many different modes for achieving resistance to apoptosis. One of them is increased signaling through the pro-survival PI3K/AKT pathway. This is usually associated with, or may produce a high expression of, the anti-apoptotic Bcl2 proteins.

For example, high expression of the anti-apoptotic Bcl2 protein induces an MDR phenotype in lymphoma cells[63]. Apoptosis is favored by low intracellular pH and impeded when it is high[64]. There is abundant evidence showing that the intracellular decrease of pH is favorable for apoptosis:

• Deoxyribonuclease II is an essential enzyme in programmed cell death and requires an acidic environment for its action[65].

• The first step in apoptosis consists of cytoplasmic acidification mainly due to proton export from mitochondria, thus alkalinizing this organelle and releasing cytochrome C[66].

• Maximal activation of caspases is only achieved with acidic cytoplasm.

• Pharmaceuticals that decrease intracellular pH induce apoptosis. This is the case of lansoprazole (inhibits the proton pump)[67], salinomycin[68], and lonidamine. The antibiotic salinomycin has shown the ability to

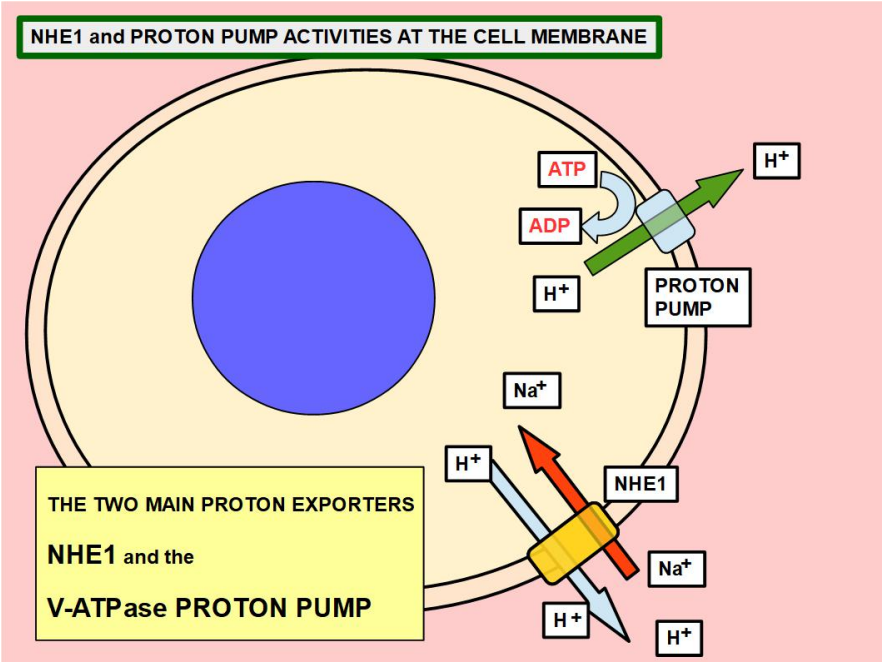

Figure 4. Increase of protons in the extracellular matrix originates mainly from the extrusion of intracellular protons by NHE1 and the proton pump.

decrease the efflux of doxorubicin as well as other chemotherapeutic drugs in MDR[69-72] through P-gp inhibition[73]. In addition to these effects, it also has anti-tumor activity independently of MDR inhibition[74-82]. However, we presume that many of the salinomycin's anticancer effects are directly related to intracellular acidification. Conversely, P-gp has antiapoptotic effects[83], and prolonged intracellular acidification decreases P-gp expression[84].

PROOF OF CONCEPT OF THE PH–MDR RELATIONSHIP

It has been known since 1990 that multidrug-resistant cells have a higher pH than their non-resistant counterparts[85]. This pH is higher on average by 0.1-0.2 and has been found in many different malignant cell lines. Although this is not a universal finding, it is quite frequent. There is also evidence that the increased intracellular pH is partly due to increased NHE1 activity.

MDR1 expressing cells were resistant to complement-mediated cytotoxicity; however, interestingly, malignant cells not expressing MDR1 that were manipulated to increase their pHi also showed similar resistance to complement-mediated cytotoxicity. Complement deposition in the cell membrane was reduced and delayed in both types of cells to a similar magnitude[86].

Belhoussine *et al.*[87] confirmed the higher intracellular pH in MDR cells. They also found that these cells had more acidified vesicles, which were more acidic than their sensitive counterparts. According to the authors, these findings suggest that non-MDR cells have a lesser ability to remove protons.

Interestingly, some publications showed the ability of amiloride, an NHE1 inhibitor and cell acidifier, to reverse both the pHi increase and MDR[88, 89].

Hamilton *et al.*[90] showed that verapamil, a classic P-gp inhibitor, lowered pHi, but they considered these two factors unrelated.

In 1997, Robinson *et al.*[91] found that the expression of MDR1 had the ability to delay apoptosis in fibroblasts transfected with the MDR1 gene and exposed to cytotoxic substances, colchicine in particular. The sole increase of pHi in non-transfected cells was able to induce a similar apoptotic delay. This finding suggests at least two things:

(1) Apoptosis requires low pHi; and

(2) One of the mechanisms used by MDR to oppose apoptosis is to maintain the increased pHi.

What remains to be seen is whether high pHi is a facilitating mechanism for resistance to apoptosis or a causal one. This report is in line with that by Weisburg[86] and supports the idea that increased pHi by itself can induce an MDR phenotype.

MDR has a "protective" effect against caspase-dependent apoptosis. Inhibition of P-gp with specific antibodies increased drug- or Fas-mediated apoptosis through caspase activation in drug-resistant cells[92], and activating caspases requires an acidic cytoplasm. Thus, protection from apoptosis seems to be a synergic activity of MDR proteins plus intracellular alkalinity.

The pH gradient inversion, with its intracellular alkalinity and without MDR proteins, has shown that it can prevent intracellular accumulation of chemotherapeutic agents. In this regard, Simon *et al.*[52] showed that intracellular alkalinity can substantially decrease accumulation and modify the intracellular distribution of weak alkaline chemotherapeutic drugs without any efflux mechanism in place. Intracellular alkalinity can prevent drugs from binding to their targets. Therefore, it is not only the extracellular ion-trapping effect that impedes the activity of weak alkaline drugs. In the experiments performed by Simon *et al.*[52], non-resistant myeloma cells had a significantly lower intracellular pH compared with myeloma chemo-resistant cells (7.1 *vs.* 7.45).

Proton pump inhibitors have shown the ability to decrease or inhibit the MDR phenotype[93]. Cisplatin treatment increased V-ATPase proton pump expression, and pHi was significantly increased in cisplatin-resistant cells. The DNA-binding ability of cisplatin was significantly enhanced in a more acidic pHi, suggesting that cisplatin's cytotoxicity was modulated by pHi. Proton pump inhibitors such as bafilomycin could synergistically increase cisplatin's cytotoxicity. These findings show that pHi is a key player in cisplatin's effects[94]. Unfortunately, bafilomycin cannot be used in clinical practice due to its toxicity.

Thiebaut *et al.*[95] confirmed the higher pHi in resistant cells compared with their non-resistant counterparts. They performed an experiment in which they raised the extracellular pH and found that non-resistant cells maintained a pHi around 7, but the resistant cells markedly increased their pHi. pHi did not increase when they treated resistant cells with P-gp inhibitors, and they suggested that P-gp also behaves as a proton exporter.

Hoffman *et al.*[96] showed that the level of resistance in MDR cells was correlated with two other parameters, namely pHi and low membrane electrical potential. Resistance was induced when they increased pHi in non-resistant cells. Furthermore, membrane depolarization also conferred a mild chemoresistance without any P-gp participation.

NHE1 is the main proton exporter, albeit not the only one, and it is overexpressed/over-active in cancer cells[97]. This is particularly so in resistant cells[97]. Cariporide is a powerful experimental NHE1 inhibitor. Interestingly, cariporide, which acidifies cytoplasm, is also able to sensitize resistant-breast cancer cells to doxorubicin[54].

NHE1 inhibition with cariporide reversed imatinib resistance in BCR-ABL-expressing leukemia cells[98-100], and NHE1 knockdown sensitized malignant cells to cisplatin-induced apoptosis[101]. Inhibiting proton extruders, among them NHE1, increased doxorubicin's cytotoxic effects on breast cancer cell lines[102].

pH regulation implies the participation of many players, in addition to NHE1. Membrane carbonic anhydrases are among these participants. Interestingly, by inhibiting membrane CAs, MDR can be reversed[103-108].

The inverted pH gradient has roles at both ends of the gradient: while cytoplasmic acidification downregulated P-gp[109], extracellular acidity upregulated it[110]. This finding explains why it is not enough to act pharmacologically on one of the components of pH deregulation; both need to be addressed.

Despite all the evidence supporting the idea that high pHi is an indispensable condition for the MDR phenotype, Young *et al.*[111] showed that increased intracellular pH is not a necessary condition for P-gp drug extrusion activity. However, we consider that this research has a conclusion bias because it only proves acid extrusion but not drug extrusion. Actually, this research showed that P-gp seems to have proton extruding abilities.

Coley *et al.*[112] found that drug resistance was related to high electric conductivity. Conductivity is not directly related to pH because it depends on the total ions (including hydrogen ions) in a solution, while pH depends only on hydrogen ions.

Mulhall *et al.*[113] showed a strong inverse relationship between conductivity and initiation of apoptosis. Thus, increased cytoplasmic conductivity seems to increase the apoptosis resistance found in resistant cells.

From these two last publications, we can deduce that the "ideal" drug-resistant cells seem to be those with:

(1) high electrical conductivity; and

(2) markedly elevated cytoplasmic pH.

These "ideal" resistant cells are refractory to apoptosis induction. We can also speculate, at this point, that increased intracellular pH is not mandatory for P-gp drug extruding activity, but it is a valuable resource for resistance to apoptosis.

Confirming the importance of conductivity, it was found that the cystic fibrosis transmembrane conductance regulator (CFTR), another member of the ABC family, decreases plasma membrane electrical potential when it is over-expressed and at the same time generates an MDR phenotype, but with a low intracellular pH. There are some similarities between this regulator and P-gp and a striking difference regarding pH[114].

NHE1 activity has been found to be increased in many tumors, and its downregulation re-sensitized cells to chemotherapy drugs[115, 116]. Drug-induced cellular surface tension modifications can impact P-gp activity[117].

Surface tension (interfacial tension) causes membrane rigidity[118]; thus, pH plays a fundamental role in this phenomenon. Furthermore, high pHi increases membrane lipid electric charges. Phosphatidylethanolamine, a normal component of the cell membrane, is involved in acid-base equilibrium with the medium.

There is abundant evidence showing that modifying cell membrane fluidity (rigidity) can reduce P-gp activity, thus reversing MDR[119-124]. Surfactants that reduce surface tension, such as Tween 80 (polysorbate 80), are able to reduce P-gp activity[125] and improve drug delivery into the cell [126].

HYPOTHESIS/THERAPEUTIC PROPOSAL

Based on the evidence discussed above, a triple approach against MDR is proposed here. This consists of a known P-gp inhibitor such as verapamil associated with a surfactant and a pH gradient reversal scheme.

Verapamil

Verapamil, a calcium channel blocker, was first found to be an inhibitor of MDR in 1981[127]. It is now a well-known P-gp inhibitor that impedes P-gp protein expression at the transcriptional level [128] and increases ATP consumption in MDR cells[129]. Direct binding of verapamil to P-gp has also been described[130]. There is abundant evidence about this drug's impact against MDR[131-137].

The surfactant Tween 80

As mentioned above, surfactants reduce cell membrane rigidity, thus counteracting one of the tools employed by MDR proteins to reject chemotherapeutic drugs. In this regard, surfactants reduce chemoresistance[138]. There is also abundant evidence concerning Tween 80's anti-MDR properties[139-142].

The pH gradient reversal scheme

This scheme is based on five drugs that target different cell membrane proteins involved in pH homeostasis and in the inverted pH gradient. Its objective is to partially downregulate all the participants in the pH gradient inversion. Full blown inhibition of all of them would be impossible without serious undesired consequences for normal cells. However, partial inhibition is possible with no toxicity. These pH modulators are:

(a) amiloride;

(b) acetazolamide;

(c) lansoprazole;

(d) quercetin; and

(e) topiramate.

The appropriate combination of these drugs creates an important decrease of the intracellular pH and at the same time increases extracellular pH. Targeting pH alterations in cancer is becoming a valid strategy in complementary treatments[143].

• Amiloride is an FDA-approved potassium-saving diuretic in clinical use for the treatment of cardiovascular diseases and is usually associated with other diuretics such as hydrochlorotiazide. Amiloride's main objective in the scheme is the inhibition of NHE1. Although it is a weak NHE1 blocker, at clinical doses, it is the only available approved drug. There are more potent NHE1 inhibitors; however, they are neither on the market nor FDA-approved. Evidence supporting amiloride's anticancer effects is abundant[144-150] and involves actions derived from its intracellular acidifying properties as well as its ability to inhibit urokinase-type plasminogen activator (uPA)[151-154]. In addition, amiloride decreases the release of tumor exosomes[155-158]. This exosome inhibition also reduces proton discharge and blocks an important pathway of cancer cell communication. Specifically, amiloride and its derivatives reversed MDR in different types of tumors[159-162].

• Acetazolamide is a nonspecific carbonic anhydrase inhibitor. Cytoplasmic pH lowering is a known effect of this diuretic[163-165] that has been in medical practice for over sixty years and is FDA approved for uses not related to cancer. There is also evidence of its ability to slow cancer growth[166,167] and inhibit MDR[168]. Furthermore, Zheng *et al.*[169] found that MDR in some tongue cancers was not produced by the three known MDR proteins of the ABC family, but rather by over-expression of CAIX. When CAIX was downregulated by antisense oligonucleotides or acetazolamide, the tumor was re-sensitized. Kopecka *et al.*[170] showed that the other membrane carbonic anhydrase, CAXII, physically interacted with P-gp on the cell surface. Silencing CAXII or inhibiting it with acetazolamide created a low intracellular pH that altered P-gp's ATPase activity and promoted chemosensitization in MDR cells. There is active ongoing research for specific CAIX and CAXII inhibitors that would make it possible to circumvent the side effects of acetazolamide[171]. For the time being, and until these new molecules are approved, we can only count on acetazolamide as a CA inhibitor.

• Lansoprazole is a vacuolar ATPase proton pump inhibitor approved by the FDA for the treatment of diseases related to excessive gastroduodenal acid production. At the cellular level, lansoprazole has the ability to inhibit proton extrusion from the cell, thus acidifying the intracellular milieu. Proton pumps can be found in intracellular membranes and the cell membrane. Those located in lysosomes keep the intra-lysosomal space acid while removing protons from the cytoplasm [Figure 5].

Lansoprazole was able to induce apoptosis in breast cancer cells[172]. Regarding MDR, lansoprazole reversed it in pets[173]. Other proton pump inhibitors, such as omeprazole, pantoprazole, and esomeprazole, showed incremental effects on different chemotherapeutic drugs[174-176]. Unfortunately, there are also negative findings, e.g., pantoprazole in a clinical trial for docetaxel in metastatic castration-resistant prostate cancer showed no effects[177], despite the favorable results in laboratory level cell tests[178-179]. Pantoprazole even increased tumor growth and decreased chemotherapeutic cytotoxicity in mice[180]. The benefits or disadvantages of pantoprazole remain controversial. According to Wang et al.[181], proton pump inhibitors increased chemosensitivity and improved overall survival and progression-free survival in patients with advanced colorectal cancer. It is possible that proton pump inhibitors are not all the same regarding their anticancer effects. This is the reason we choose lansoprazole, which is less controversial than pantoprazole. Proton pump inhibitors decreased cisplatin sequestration in endosomes that were finally released from the cell in a melanoma model[182]. Luciani *et al.*[183] treated malignant cells with proton pump inhibitors, improving the accumulation of intracellular cytotoxic drugs. Intermittent proton pump inhibitors associated with standard chemotherapy administration improved the clinical outcome in metastatic breast cancer patients[184].

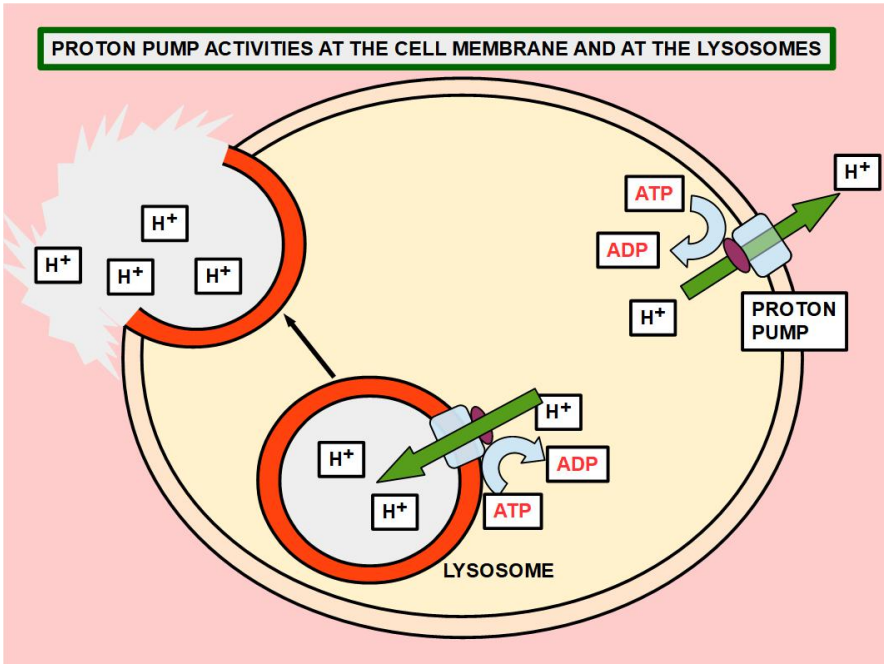

Figure 5. Different arrangements of proton pumps in the cell membrane and lysosomes. While in the cell, membrane the pump extrudes protons towards the extracellular space, in the lysosome, it pumps the protons into it. In a further step, the lysosome releases protons into the matrix. The functional end result is the same in both cases: inversion of the pH gradient.

• Quercetin is a natural flavonoid that is not approved as a drug by the FDA but is available as an over-the-counter nutritional supplement. However, it is the only compound that can be found on the market with a strong ability to inhibit monocarboxylate transporters[185, 186]. MCT inhibition by quercetin induces important intracellular acidification[187]. Significantly, there is considerable evidence of its capacity to reverse the MDR phenotype[188-218]. Despite this large body of evidence, absolute lack of toxicity, and low cost, we could not find clinical trials exploring the substance's ability for MDR reversal. Adverse events using high levels of quercetin (1 g daily) as a nutritional supplement have been rarely reported[219]. Quercetin also has additional beneficial effects in cancer:

(a) Inhibition of the PI3K/Akt/mTOR pathway[220, 221];

(b) Proteasome inhibition that leads to mTOR inhibition and autophagy[222, 223];

(c) Decrease of ROS that diminishes PKC activity[224];

(d) Cancer cell-specific inhibition of the cell cycle[225, 226];

(e) Downregulation of heat shock protein 90[227];

(f) Inhibition of β-catenin signaling[228];

(g) Inhibition of pleiotropic kinases[229]; and

(h) Induction of apoptosis[230].

• Topiramate is an FDA-approved drug for the treatment of seizures and epilepsy. It has four pharmacological effects that can benefit MDR reversal: (1) carbonic anhydrase inhibition; (2) intracellular acidification; (3) inhibition of voltage-gated sodium channels; and (4) inhibition of aquaporin 1[231-235]. There are no publications on topiramate having direct effects on MDR; however, refractory epilepsy in rats has been found to be associated with increased expression of P-gp[236, 237]. Topiramate and other anticonvulsants are substrates for P-gp. Although there is no empirical proof, we suspect that topiramate may saturate P-gp extrusion capacity. The reason for including topiramate in the scheme is mainly for two of its effects: cytoplasmic acidification and voltage-gated sodium channel inhibition.

• Statins are inhibitors of the *de novo* synthesis of cholesterol by blocking hydromethyl glutaryl coenzyme A reductase, an enzyme that is a rate-limiting factor for mevalonate synthesis and the mevalonate pathway. Therefore, statins decrease endogenous cholesterol production. Cell membrane rigidity depends on the amount of cholesterol, among other factors.

DISCUSSION

pH homeostasis is a complex mechanism in which different transporters, exchangers, channels, and enzymes are involved in overlapping proton and ionic trafficking between different cellular compartments. The results of these ionic movements lead to the best possible pH balance for cellular functions. Tumor pH homeostasis is different from that found in normal tissues, and this difference involves a proliferative and progressive advantage for the malignant phenotype. Each enzyme in a complex organism has a specific pH in which it works at the optimum speed and capacity. This is the pK. Tumors, by creating a different pH homeostasis, are signaling which enzymes should be more active and when, thus regulating tumor metabolism.

This essentially means that pH is a signaling molecule. If anyone doubted that pH is a molecule, he or she would be right. It is not a molecule but many molecules, or, even better, many protons. The cell behavior is thus conditioned by the number of protons present. The MDR phenotype shows a slight difference with the drug-sensitive one: a higher intracellular pH. This difference allows for two characteristics of the MDR cell:

(1) A more rigid cell membrane that plays a role in impeding cytotoxic drug access inside the cell; and

(2) A higher resistance to apoptotic signals.

A third characteristic must be added to this: the ion trapping produced by the strongly acidic extracellular matrix.

Multidrug resistance is not a one-protein job. At a certain point, P-gp and its sister molecules of the ABC family require adequate cell membrane rigidity, higher apoptosis resistance, and more ion trapping, whether in the matrix or inside lysosomes. This means the appropriate pH. MDR seems to function better with a high intracellular pH. This does not mean that one is the cause of the other. An MDR phenotype can be achieved even with low intracellular pH; this is the case of CFTR. Conversely, a high pHi can generate an MDR phenotype without over-expressing the MDR proteins.

This evidence hints towards the idea that high pHi and the MDR proteins complement each other, rather than there being a causal relationship. Both come together in one characteristic of the resistant cell: increased cell membrane rigidity. High pHi induces membrane rigidity, which in turn cooperates with MDR.

All this said, it becomes evident that for a successful fight against MDR, it is not enough to downregulate P-gp, etc., but pH and cell membrane rigidity must be tackled as well. The scheme proposed here confronts the three issues:

• P-gp with calcium channel blockers such as verapamil or others;

• pH gradient inversion with the pH-centered treatment; and

• membrane rigidity with surfactants such as Tween 80 and others.

The MDR problem has even further complexities. Balza *et al.*[238], working with two different breast cancer cells, a triple-negative one and a hormone-sensitive one, found that:

(1) Associating cisplatin with an amiloride derivative was significantly more effective than treatments with cisplatin plus esomeprazole in triple-negative cells; and

(2) Esomeprazole alone was more effective in hormone-sensitive cells.

This shows that pH-centered treatments as complementary therapy may differ according to the type of cell.

Importantly, persistent intracellular acidification was able to downregulate the MDR phenotype[84].

Figure 6 is a summary of concepts discussed in this paper, while Figure 7 shows the site of action of MDR inhibitors.

It is important to note that extracellular acidity per se can induce P-gp expression. Figure 6 shows that the ABC family of drug extruders, intracellular alkalosis, and extracellular acidosis can all generate an MDR phenotype in an independent manner. However, there is evidence supporting the relationships among these three factors. Increased extracellular acidity induces P-gp expression. MDR cells with increased P-gp expression usually show increased intracellular alkalinity[87, 95]. This, in turn, prevents apoptosis and increases cell membrane rigidity[112, 113], creating the ideal environment for drug resistance[117].

CONCLUSIONS

MDR represents the last chapter of chemotherapeutic cancer treatment. It leaves the oncologist on a very narrow path to continue patient care. Unfortunately, there is no accepted treatment protocol. In this review, we propose a multidrug approach that simultaneously targets three important MDR characteristics, namely the MDR proteins, dysregulated pH, and cell membrane rigidity, with a rationally constructed approach.

This scheme has not been tested on clinical grounds. However, each of its components has separately provided successful experimental results, with the exception of topiramate, which has not been tested in the MDR context. This justifies their combination, as each of them targets different aspects of the MDR

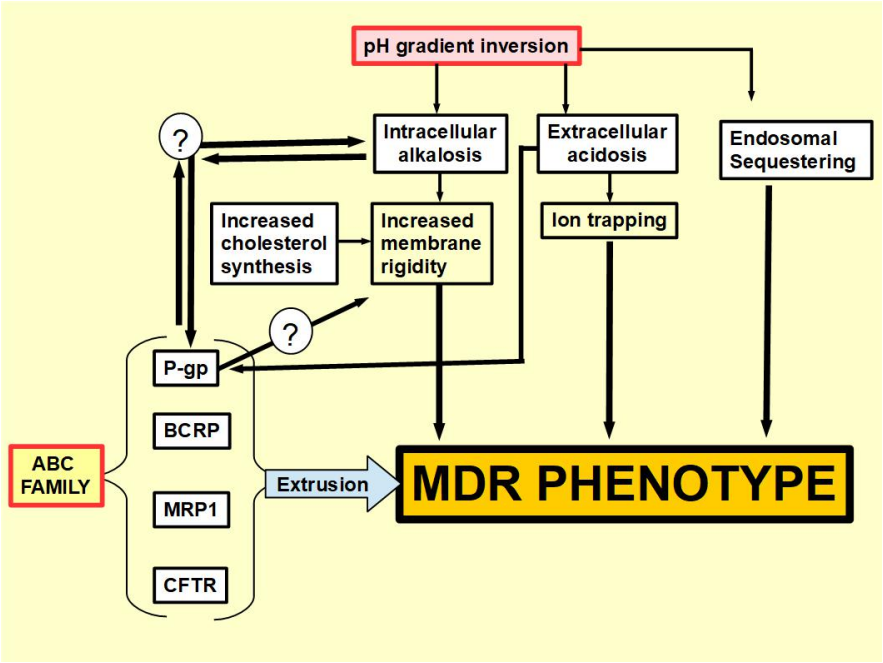

Figure 6. A synthesis of known and unknown relationships between pH and the MDR phenotype. Based on references cited above (Ref.[48-50,239-241]). Multiple drug resistance (MDR)

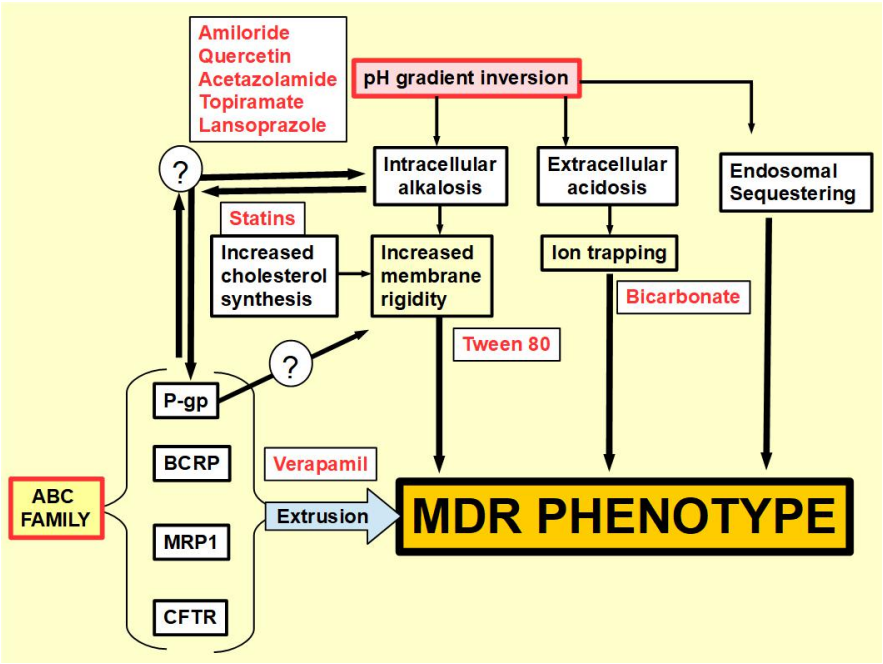

Figure 7. Site of actions of the anti-MDR scheme. Reversion of the deregulated pH gradient is able to act against the MDR phenotype in two ways, by decreasing extracellular acidity and reducing intracellular pH. MDR: Multiple drug resistance.

conundrum. Well-planned clinical trials are needed to evaluate this proposal. Furthermore, the scheme has almost no toxicity for normal cells, and there is ample clinical experience with the use of all these drugs.

DECLARATIONS

Authors' contributions

The author contributed solely to the article.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Mechanisms of chemotherapy resistance in ovarian cancer

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Abstract

Ovarian cancer is one of the most lethal gynecologic cancers. The standard therapy for ovarian cancer has been the same for the past two decades, a combination treatment of platinum with paclitaxel. Recently, the FDA approved three new therapeutic drugs, two poly (ADP-ribose) polymerase inhibitors (olaparib and niraparib) and one vascular endothelial growth factor inhibitor (bevacizumab) as maintenance therapies for ovarian cancer. In this review, we summarize the resistance mechanisms for conventional platinum-based chemotherapy and for the newly FDA-approved drugs.

Keywords: Drug resistance, ovarian cancer, cisplatin, carboplatin, paclitaxel, olaparib, niraparib, bevacizumab

INTRODUCTION

Ovarian cancer is one of the most lethal gynecologic cancers, with projected 21,410 cases and 13,770 deaths in the United States in 2021[1]. The standard treatment for ovarian cancer is platinum-based chemotherapy (carboplatin or cisplatin) in combination with paclitaxel, and it has remained the same for the past two decades. Most patients are initially responsive to these treatments; however, relapse occurs in around 80% of women due to platinum resistance, causing the need to comprehend its molecular mechanisms to improve

treatment efficacy and patient survival[2].

Cisplatin was first synthesized in 1844 by Michele Peyrone, but it was not until 1965 that Barnett Rosenberg from Michigan State University discovered that cisplatin inhibits cell division. Since then, cisplatin has been widely used for the treatment of bladder, lung, head and neck, testicular, and ovarian cancers[3]. In 1978, it became the first FDA-approved platinum-based compound for cancer treatment[4]. Subsequently, a second-generation platinum-drug, carboplatin, was developed and became FDA-approved in 1989[5,6].

In the 1960s, the National Cancer Institute and the U.S. Department of Agriculture collaborated to identify potential cytotoxic anticancer properties from 115,000 plant extracts[7]. From screening these samples, Arthur Barclay identified cytotoxic properties in bark extract from the Pacific yew tree, *Taxus brevifolia*,in 1964. Three years later, the active ingredients from *Taxus brevifolia* were identified and named as taxol. A fully synthetic version of the drug, called paclitaxel, was FDA-approved for use as a chemotherapeutic agent for ovarian cancer in 1992. Clinical trials in the early 2000s have shown improved outcomes in women with relapsed ovarian cancer when treated with paclitaxel in addition to platinum-based chemotherapy[8]. Since then, the standard treatment of ovarian cancer patients continues to be platinum-based chemotherapy in combination with paclitaxel.

Recently, there have been new advancements in treatment recommendations for ovarian cancer patients with the emergence of three new FDA-approved chemotherapeutic drugs. In 2018, the first poly (ADP-ribose) polymerase inhibitor (PARPi), olaparib, was FDA-approved as maintenance therapy for ovarian cancer, followed by the approval of niraparib (another PARPi) in 2020. In 2020, a vascular endothelial growth factor inhibitor (VEGFi), bevacizumab, was also FDA-approved as another maintenance therapy for ovarian cancer. In this review, we will discuss the resistance mechanisms of conventional chemotherapies and provide insights into the resistance mechanisms against the recently FDA-approved chemotherapeutic drugs for ovarian cancer.

MECHANISMS OF RESISTANCE TO PLATINUM AGENTS

Cisplatin and carboplatin target cancer cells by forming adducts/crosslinks with DNA purine bases, with a preference for guanine. These crosslinks result in DNA damage that impedes proper genome replication, transcription, and triggers cell apoptosis[9]. A prevalent resistance mechanism centers around inhibiting the compound from reaching the DNA, which is mediated by efflux transporters. In addition, once DNA lesions are formed, DNA repair pathways are activated to fix DNA damage caused by the platinum agents, as described below [Figure 1].

Influx and efflux transporters

One of the most widely accepted mechanisms of platinum resistance is the dysregulation of both influx and efflux pumps/transporters, which modulates the transport of platinum in ovarian cancer cells. When the first multidrug resistance transporter, an ATP-binding cassette transporter (also called ABCB1, P-glycoprotein, P-gp, or MDR1), was identified to have a function in the efflux of anticancer drugs out from cancer cells, it was also tested whether cisplatin is pumped out by P-gp[10-13]. However, it was identified that cisplatin is not a direct substrate of P-gp. Thus, it prompted the investigators to identify transporters that are similar to P-gp but have the ability to efflux cisplatin. This led to the discovery of the influx transporter, copper transporter 1 (CTR1), and efflux transporters, ATPase copper-transporting alpha and beta (ATP7A and ATP7B). Subsequent studies have shown that cisplatin-sensitive A2780 ovarian carcinoma cells have higher CTR1 expression than cisplatin-resistant A2780CP[14] and that overexpression of CTR1 in A2780 cells increased the influx of cisplatin[15]. Clinically, patients who had cytoreductive surgery followed by platinum-

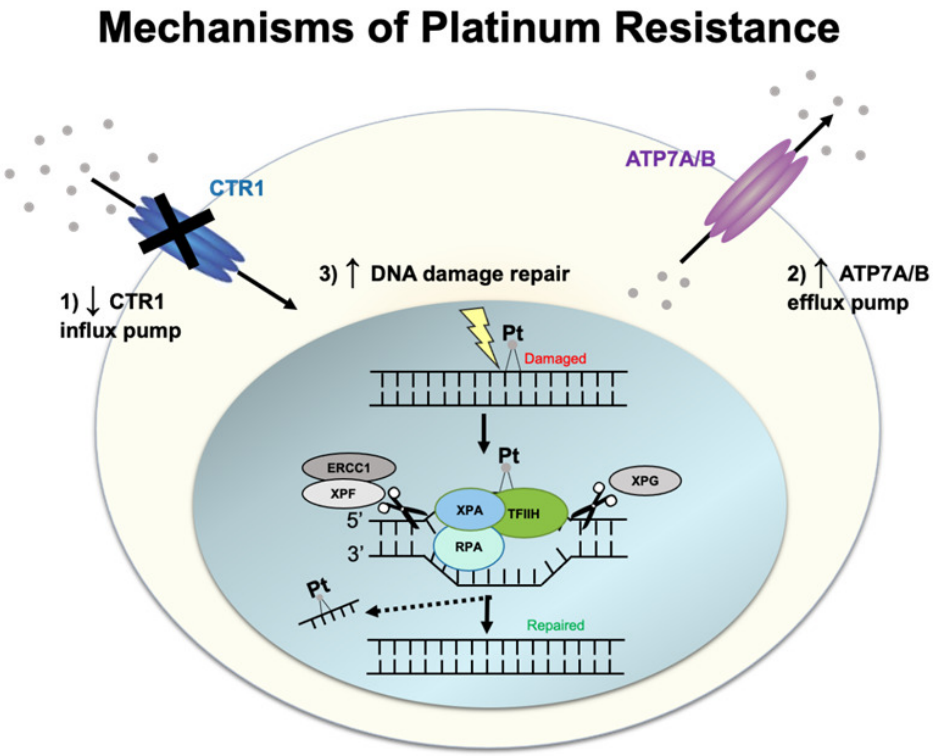

Figure 1. Schematic illustration of platinum resistance.

based chemotherapy had CTR1 mRNA levels that correlated with platinum sensitivity[16]. Interestingly, cisplatin exposure in A2780 cells triggers rapid downregulation of CTR1, thereby inhibiting further cisplatin accumulation in the cells. This negative feedback loop has been proposed to result in resistance to cisplatin[17]. Cisplatin-induced downregulation of CTR1 is inhibited by the proteasome inhibitor bortezomib[18]. A clinical trial (NCT0107441) was recently completed to determine intraperitoneal bortezomib's maximum-tolerated dose and dose-limiting toxicities when administered with carboplatin in an epithelial ovarian, fallopian tube, or primary peritoneal cancer.

On the other hand, ATP7A/B efflux platinum[19,20]. However, only the silencing of ATP7B, and not in the silencing of ATP7A, in cisplatin-resistant cells (A2780-CP20 and RMG), resulted in increased sensitivity to cisplatin[21]. Clinical studies have also supported the prognostic value of ATP7B in ovarian cancer patients treated with cisplatin therapy[22,23]. In addition, a clinical study on 152 ovarian cancer patients has shown that genetic polymorphism in ATP7A is implicated in cisplatin resistance, and genetic polymorphisms in CTR1 is implicated in carboplatin resistance[24].

DNA repair

After entry of platinum drugs into the cytoplasm via CTR1, the platinum drugs enter the nucleus, where they form intrastrand and interstrand crosslinks with the DNA. Resistance to platinum occurs via DNA repair pathways. Single-strand DNA lesions are repaired by nucleotide excision repair (NER), and double-strand lesions are repaired either by homologous recombination (HR) or non-homologous end-joining (NHEJ) pathway[25]. In the NER pathway, the site of the DNA lesion is cleaved by ERCC1-XPF and XPG endonucleases to remove the DNA lesion. High ERCC1 expression is associated with platinum resistance in epithelial ovarian cancer but is not associated with patient survival[26]. Promoter methylation of mismatch

repair (MMR) genes can also lead to cisplatin resistance by downregulating MMR-driven DNA damage response[27]. The MMR pathway carries DNA repair during DNA replication and recombination and specifically recognizes mismatched base pairing, insertions, and deletions[28]. In a study using cisplatin-resistant and MMR-deficient ovarian tumor xenografts caused by MLH1 promoter hypermethylation, treatment with the demethylation agent, 2'-deoxy-5-azacytidine, was shown to improve response to cisplatin and carboplatin[27].

Emerging studies: other resistance mechanisms to platinum agents

More recently, other examples of platinum resistance have been explored. These include upregulating of de-ubiquitination of proteins targeted for proteasomal degradation[29], increased cisplatin-induced autophagy[30], and dependence on mitochondrial oxidative phosphorylation for energy supply[31]. Metabolic reprogramming and angiogenesis are hallmarks of cancer[32] that provide tumor supply of nutrients and oxygen, energy efficiency, and drive cell survival; thus, they are also speculated to be involved in chemotherapy resistance. For instance, it has been recently shown that upregulation of the serine/threonine kinase Aurora-1 in cisplatin-resistant ovarian cancer increases glycolysis and suppresses cell senescence by stimulating the transcription factor sex determining region Y-box 8 (SOX-8)[33]. Another study has shown that fibrillin-1 (FBN1) is significantly upregulated in cisplatin-resistant ovarian cancer organoids and tissues and that FBN1 drives phosphorylation of VEGF2 and nuclear translocation of the transcription factor signal transducer and activator of transcription 2 (STAT2), which affects the expression of genes associated with STAT2-mediated glycolysis and angiogenesis[34]. A combination of FBN1 knockout and an antiangiogenic drug was demonstrated to improve cell sensitivity to cisplatin. Altogether, there are several emerging studies on mechanisms of platinum resistance in ovarian cancer.

MECHANISMS OF RESISTANCE TO PACLITAXEL

Paclitaxel exerts its effect by binding to the β-subunit of tubulin and causing tubulin polymerization in the absence of GTP, a factor that is normally required for microtubule polymerization[35]. Once bound, paclitaxel stabilizes microtubules and prevents tubulin depolymerization. This inhibits the shortening of the microtubules during anaphase when it is necessary to pull apart sister chromatids, which results in cancer cell death[36]. Below, we discuss several mechanisms of resistance to paclitaxel [Figure 2].

Efflux by P-glycoprotein

Like platinum-based chemotherapies, cancer cells can develop resistance to paclitaxel via efflux of paclitaxel out from the cancer cells. However, unlike cisplatin, paclitaxel is the major substrate of P-gp[11,37]. In order to prevent P-gp mediated efflux of paclitaxel, recent studies have shown that mutations in P-gp can suppress the efflux of paclitaxel[38], and substitution of 14 conserved residues in homologous transmembrane helicases 6 and 12 with alanine resulted in reversal of P-gp from efflux to influx pump[39], a potential future avenue for preventing efflux of paclitaxel.

Tubulin isotype composition

Paclitaxel is involved in the stabilization of microtubules, and as such, changes in microtubule composition can play a key role in determining cell susceptibility to paclitaxel. While tubulin has multiple isoforms, microtubules composed of mostly the βIII isoform show significantly lower stability compared to βI and βII microtubules[40]. Lower microtubule stability can counteract the microtubule-stabilizing effect of paclitaxel and thus allow cell division to occur despite paclitaxel activity. Paclitaxel-resistant ovarian tumors have been sampled to reveal up to a 4-fold increase in the proportion of βIII isoform, indicating that upregulation of β III isoform production may confer paclitaxel resistance[41].

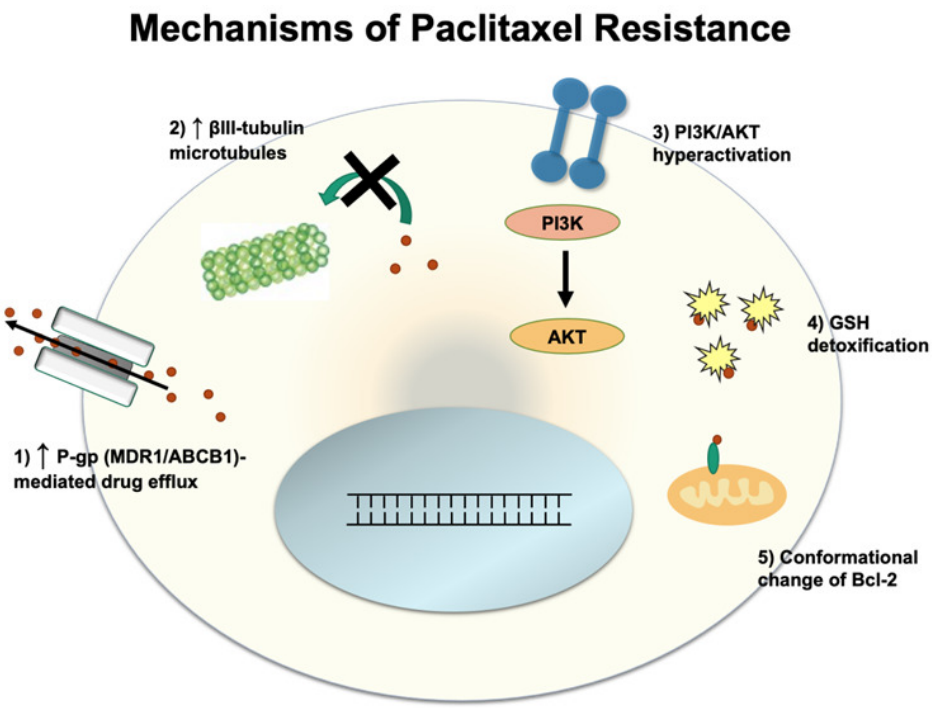

Figure 2. Schematic illustration of paclitaxel resistance.

Phosphoinositide 3-kinase /protein kinase B pathway

The Phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway has been shown to be a key driver of metastasis and drug resistance in many different cancers, including ovarian cancer. Overactivation of this signaling pathway leads to the upregulation of factors involved in cell proliferation and migration. Mechanisms of PI3K/AKT hyperactivation include loss of function in PTEN (a negative regulator of PI3K/AKT), mutations in PI3K that confer constitutive activity, and hyperactivity of AKT[40]. These mutations cause the activation of pro-mitotic factors that overpower the anti-proliferative signals that result from paclitaxel dosing.

Glutathione S-transferase 1

The detoxifying powers of the glutathione pathways are turned against paclitaxel in this resistance mechanism. In healthy cell conditions, Glutathione S-transferase P1 (GSTP1) production is inhibited by high PRMT6 activity, which inhibits the production of the GSTP1 precursor, G6PD. In this particular resistance mechanism, PRMT6 is downregulated in cancer cells, allowing for increases in G6PD production and higher levels of GSTP1 as a result. Thus, paclitaxel becomes sequestered by GSTP1 and detoxified in the cells before it can bind tubulin[42]. In the A2780 ovarian cancer cell line model, GSTP1 knockdown suppressed the invasion and migratory properties of the cells and sensitized the cells to cisplatin and carboplatin[43].

B-cell lymphoma 2 family

In the B-cell lymphoma 2 (Bcl-2) family, there are the pro-apoptotic factors, BAD and BAX, as well as the anti-apoptotic factors, Bcl-2, Bcl-XL, and Mcl-1[44]. Paclitaxel has been found to alter Bcl-2 activity, transforming it into a pro-apoptotic factor[45]. Mechanistically, paclitaxel mimics the nuclear orphan receptor Nurr77 to cause this effect. The Nurr77 and paclitaxel have structural similarities that explain this mimicry[46]. Just like Nurr77, paclitaxel binds to the N-terminal loop of Bcl-2 and induces a conformational

change to expose the Bcl2-homology 3 domain. Upon phosphorylation of this conformational isoform, Bcl-2 changes from an anti-apoptotic factor to a pro-apoptotic factor by inducing cytochrome c release from the mitochondria[47]. Resistance to paclitaxel can be attributed to increases in anti-apoptotic Bcl-2 family members, as the anti-apoptotic factors inhibit FasL production, a ligand involved in cell death, by preventing its gene transcription[44,48].

In addition, genetic differences in Bcl-2 have been attributed to varying rates of paclitaxel treatment success in patients. Recent studies have identified a T>C variant (RefSNP rs1801018) in the Bcl-2 sequence that is highly associated with paclitaxel resistance in multiple tumor types. In a retrospective genomic analysis of cancer patients, 73% of patients with T at location 21 did not respond to the platinum-paclitaxel combination therapy[49]. Although the exact mechanism facilitating this resistance is currently unknown, the data indicate a vital relationship between the Bcl-2 sequence and paclitaxel susceptibility and resistance.

Paclitaxel and cisplatin cross-resistance

Cisplatin and paclitaxel are commonly used in conjunction since the two drugs bring cytotoxicity by distinct mechanisms and, thus, provide two unique barricades against uncontrolled cell growth[9]. Yet, as cells become resistant to cisplatin, they can also become resistant to paclitaxel. One such mechanism of this is through upregulation of cell survival pathways. Cells can combat cisplatin- and paclitaxel-induced apoptosis by upregulation of cell survival pathways, such as TNF/NFκB[50]. It is not yet known whether paclitaxel resistance mechanisms are able to confer cisplatin resistance, but the existence of common resistance mechanisms between the two drugs posits the idea.

MECHANISMS OF RESISTANCE TO PARP INHIBITORS

Olaparib and niraparib are two PARPis recently approved by the FDA for targeted therapy in ovarian cancer in 2018 and 2020, respectively. These drugs are currently used as maintenance therapies or advanced treatments of ovarian cancer in platinum-sensitive patients who have undergone chemotherapy as first-line chemotherapy[51]. PARPi undermines single-strand DNA (ssDNA) damage repair, more specifically the base excision repair pathway, either by trapping PARP proteins on the DNA site of the lesion or by blocking PARP catalytic domain[52]. This prevents the binding of NAD+, a cofactor necessary for the post-translational modification, named PARPylation, onto targeted proteins to happen[53]. Unrepaired ssDNA breaks lead to double-strand DNA (dsDNA) breaks and genomic instability, triggering cell death[54]. Approximately 30 - 60% of the patients treated with PARPi respond to the treatment[55]; however, several cases of resistance have been reported. As the first FDA-approved PARPi, olaparib is the most investigated in terms of resistance mechanisms and will be further discussed below [Figure 3].

DNA repair

Patients with impaired homologous recombination (HR) repair machinery often respond better to PARPi treatment. This combination leads to the accumulation of dsDNA breaks due to deficient DNA repair[56]. Hence, most resistance to olaparib and other PARPi has been linked to restoring DNA repair activity. Recently, it was shown that olaparib-resistant epithelial ovarian cancer cells harboring BRCA2 mutation had augmented microhomology-mediated end joining (MMEJ) pathway, which allows the cells to overcome PARP inhibition[57]. The increased expression of the epigenetic reader Bromodomain-containing protein 4 (BRD4) induces upregulation of aldehyde dehydrogenase 1 family member A1 (ALDH1A1), an enzyme associated with chemotherapy resistance through drug metabolism and inhibition of apoptosis signaling mediated by reactive oxygen species (ROS). ALDH1A1, in turn, is proposed to stimulate MMEJ and dsDNA repair. Thus, ALDH1A1 specific inhibition was shown to recover PARPi sensitivity. In another study, BRCA2-mutated high-grade serous ovarian carcinoma cells (HGSOC) resistant to olaparib exhibited

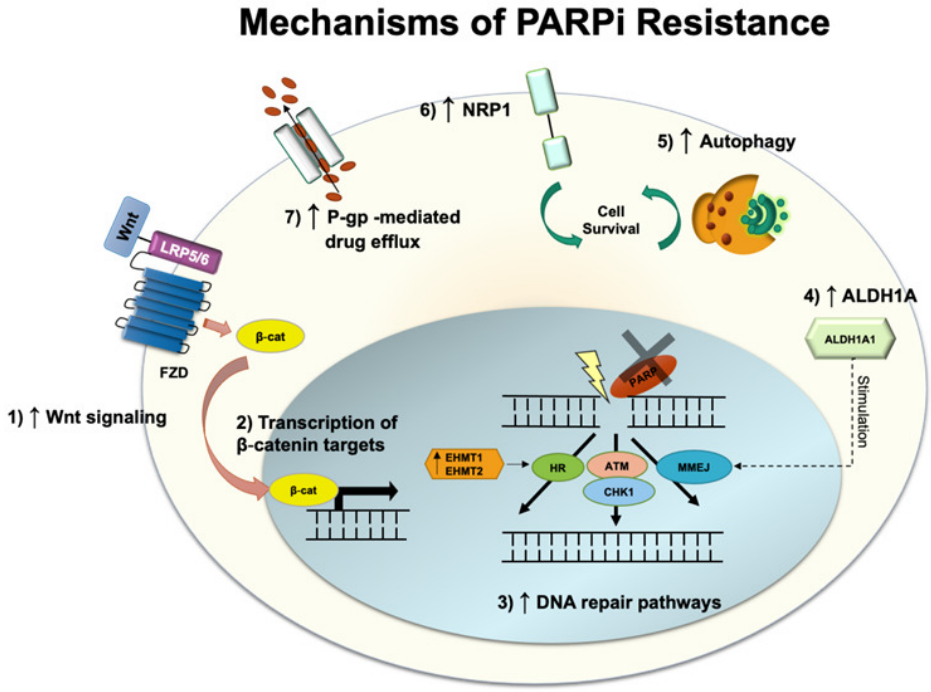

Figure 3. Schematic illustration of PARPi resistance. PARPi: Olymerase inhibitor.

histone-lysine-N-methyltransferase 1 and 2 (EHMT1/2) overexpression and consequent increase in histone H3 lysine 9 dimethylation, which was correlated with poorer overall survival[58]. As EHMT1/2 has been demonstrated to play a role in DNA repair by directly recruiting DNA damage response factors, the interruption of its activity leads to HR and NHEJ impairment, and disruption of the cell cycle, sensitizing these cells to PARPi. Nonetheless, no increase in cell death rates was observed when EHMT1/2 was inhibited. An alternative mechanism of resistance to olaparib in HGSOC cell lines independent of BRCA reverse mutation was demonstrated to be associated with increased activation of the Wnt signaling pathway[59]. Although Wnt drives different cell pathways by directly promoting β-catenin accumulation and upregulating β-catenin target-gene transcription, treatment with its inhibitor, pyrvinium pamoate, and olaparib abrogated DNA damage repair[59]. Furthermore, olaparib-resistant cell lines were shown to have enhanced HR and distal NHEJ induced either by Wnt-dependent or independent mechanisms.

Cell cycle

The cell cycle regulation is critical for the proper execution of DNA repair[60]. Upon recognition of DNA damage, checkpoint kinases ATM and ATR phosphorylate downstream signaling pathways to determine whether to continue with cell cycle progression or pause for DNA repair[61]. HGSOC has universal p53 loss, which causes dysfunctional G1/S checkpoint, which makes the tumor be dependent on G2/M cell cycle arrest for DNA repair[62]. This G2/M cell cycle arrest is mediated by the cell cycle checkpoint kinase 1 (Chk1) and is activated by the DNA replication stress marker ATM and ATR, allowing HR to fix dsDNA breaks in the presence of collapsed replication forks. Moreover, Chk1 phosphorylates BRCA2 and RAD51 recombinase, assisting their nuclear translocation and interaction. RAD51, in turn, mediates invasion and pairing of the broken DNA strand with the homologous chromosome, used as the template for DNA recombination and repair[63,64].

Inhibition of Chk1 with prexasertib has been demonstrated to attenuate olaparib-induced RAD51 translocation to the nucleus and foci formation leading to HR deficiency in BRCA wild-type or BRCA2-restored function HGSOC[62]. Thus, Chk1 inhibition was shown to enhance the sensitivity of ovarian cancer cells to DNA damage response inhibitors, such as PARPi, and the combination of both drugs decreases cell viability due to increased DNA damage and apoptosis. In addition, it promotes cell cycle progression to the M phase independent of the BRCA phenotype in cells once arrested in the G_2/M checkpoint by olaparib treatment only. Importantly, over 90% of HGSOC have abnormal function of the tumor-suppressor TP53[65], meaning that cells rely on the G_2/M checkpoint in the absence of the G_1/S. The combinatory treatment forces the cell to enter the M phase even in the presence of unrepaired DNA breaks, leading to cell death.

Efflux transporters

Apart from DNA damage repair-associated mechanisms, other models were proposed to elucidate ovarian cancer resistance to olaparib. One such example is drug efflux pumps, and they have been widely explored to be involved in multi-drug resistance mechanisms. P-gp, which is encoded by *ABCB1* gene, is associated with first-line chemotherapy resistance to paclitaxel and other taxane drugs[13]. *ABCB1* gene expression and copy number were shown to be increased in paclitaxel-resistant ovarian cancer cells presenting cross-resistance to olaparib, and both drugs were actively effluxed from the cells[66]. Inhibition of P-gp with elacridar was able to resensitize the resistant cells to olaparib and paclitaxel, hence a combinatory treatment to improve sensitivity to PARPi. Similarly, targeting neuropilin-1 (NRP1), a transmembrane receptor that contributes to cell contact evasion and tumorigenesis in ovarian tumors, with the miRNA miR-200c, induces sensitization of resistant SKOV3 ovarian cancer cell lines (BRCA wild-type) to olaparib[56]. In these cells, NRP1 was demonstrated to be present in higher levels compared to the sensitive UWB1.289 BRCA1-null cell line or the partially resistant UWB1.289 in which BRCA1 function was restored.

Autophagy

Regardless of the BRCA phenotype, clinical trials (NCT01847274; NCT02354586) have demonstrated that niraparib leads to better outcomes in patients with wild-type HR[67]. Although niraparib resistance in ovarian tumors has been reported, whether similar pathways drive this as for other PARPi remains to be elucidated. As the FDA approval of niraparib happened more recently, only a few studies have explored its specific resistance mechanisms to date. Nonetheless, a recent study treating a variety of ovarian cancer cell lines, including the BRCA wild-type OVCAR8 and HEY cells, with four PARPis, olaparib, niraparib, rucaparib, and talazoparib, demonstrated increased autophagy activation[68]. Autophagy has been described as a common resistance mechanism to overcome anticancer drugs by providing the energy supplies necessary for cell survival under stressful conditions. It also contributes to the hypoxic microenvironment and metabolic stress, two components modulated by cancer cells to escape cell death. Hence, targeting autophagy for downregulation may improve patient response to PARPi. Combining olaparib and inhibitor that can target autophagy may increase ATP phosphorylation and ROS formation. As a consequence, increased cell death and proliferation suppression were observed after the combinatory treatment. Further studies are needed to elucidate whether autophagy itself is enough to drive cell survival in cells containing high genomic instability.

In summary, ovarian cancers have several resistance mechanisms to PARPis. These include stimulating DNA damage response, preventing genomic instability either by upregulating Wnt signaling, inducing EHMT1/2 overexpression, or increasing ALDH1A1 levels. Alternatively, olaparib might be actively effluxed from the cells using the P-gp pump. Resistance to olaparib and niraparib has also been attributed to PARPi-mediated-autophagy, which contributes to the metabolic modulation of cancer cells and prolonged cell survival.

MECHANISMS OF RESISTANCE TO BEVACIZUMAB

Bevacizumab is a humanized monoclonal IgG1 antibody designed to target VEGF[69] and was recently approved as maintenance therapy for ovarian cancer in 2020. VEGF, an angiogenic growth factor, is often produced by cancer cells to drive blood vessel growth and, consequently, divert nutrients directly to tumors[70,71]. Bevacizumab binds directly to circulating VEGF, preventing it from interacting with VEGF-receptors, thus inhibiting angiogenesis by starving the tumor from nutrients. Resistance mechanisms against bevacizumab have been discussed in other cancer settings[72,73]. Here, we discuss resistance mechanisms to bevacizumab in ovarian cancer [Figure 4].

Bevacizumab-induced decreased antibody uptake, increased vessel pericyte coverage, and angiogenesis

When angiogenesis occurs in solid tumors, it forms a defective vasculature with increased tumor permeability. This alters the tumor microenvironment and affects intra-tumoral drug delivery. When ovarian cancer SKOV3 mouse xenograft was evaluated for antibody uptake using PET imaging of ^{89}Zr-bevacizumab, it was observed that bevacizumab treatment decreased tumor uptake and lessened intra-tumoral accumulation of bevacizumab with increased vessel pericyte coverage[74]. Pericyte promotes endothelial cell survival via activation of VEGF-A, and, therefore, may contribute to the resistance to VEGFi[75,76]. In addition to increased vessel pericyte coverage, ovarian cancer cells can circumvent bevacizumab via angiogenesis. For instance, the crosstalk between endothelial cells and ovarian cancer cells can activate the PI3K/Akt pathway and stimulate the proangiogenic factor FGF2, overcoming VEGF-dependent vascularization as an evasive mechanism[77].

Ephrin type-B receptor 4

Ephrin type-B receptor 4 (EphB4) is a tyrosine kinase receptor with a functional role in blood vascular morphogenesis and angiogenesis[78]. While the exact resistance mechanism has not yet been determined, EphB4 is overexpressed in bevacizumab-resistant ovarian cancer SKOV3 xenograft and co-administration of bevacizumab with the EphB4 blocker, NVP-BHG712, results in reversal of resistance and inhibition of tumor growth[79].

The mechanisms by which cancer cells develop resistance to bevacizumab have yet to be fully characterized. While the resistance mechanisms mentioned above have been identified, the future discovery of additional resistance mechanisms should be anticipated in the ovarian cancer setting.

CONCLUSION

In this review, we have described mechanisms of drug resistance against platinum, paclitaxel, olaparib, niraparib, and bevacizumab in the context of ovarian cancers. While we know many resistance mechanisms against platinum and paclitaxel, we continue to discover novel resistance mechanisms due to technological advancement in the field. There is currently a major interest in the field to understand the resistance mechanisms of new ovarian cancer treatments such as olaparib, niraparib, and bevacizumab. We anticipate more new insights and discoveries in novel resistance mechanisms as well as novel approaches to covercome drug resistance (i.e., nanomedicine[80]) in the next few years.

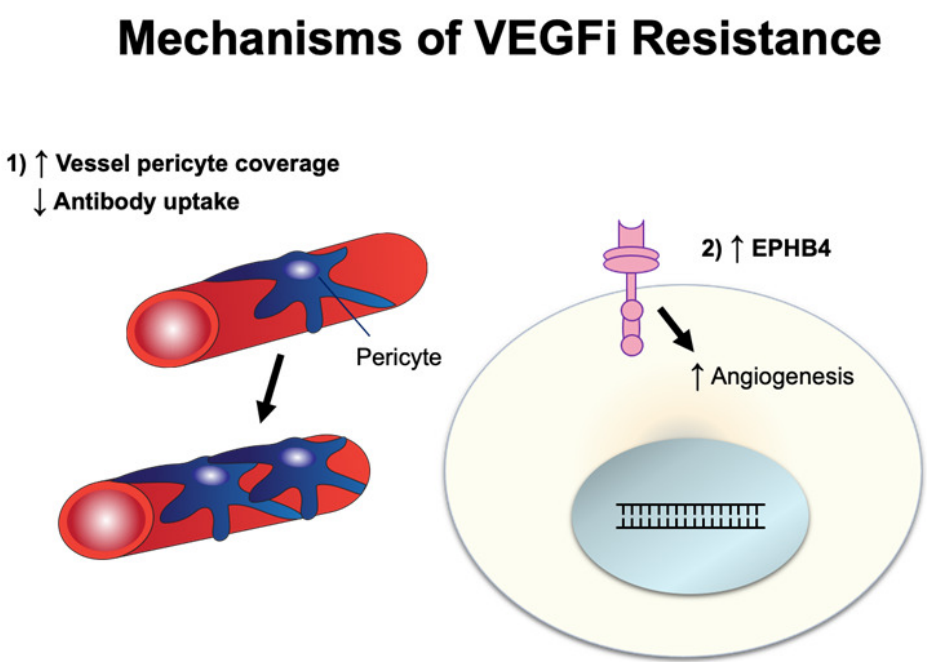

Figure 4. Schematic illustration of VEGFi resistance. VEGFi: Vascular endothelial growth factor inhibitor.

DECLARATIONS

Authors' contributions

Ortiz M wrote the resistance mechanisms to platinum, olaparib, niraparib and generated the figure; Wabel E wrote the paclitaxel and bevacizumab resistance mechanism and generated the figure; Mitchell K wrote the platinum resistance mechanisms; Horibata S supervised the work, generated figures, edited, and finalized the manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interesst

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Targeting the complement system in pancreatic cancer drug resistance: a novel therapeutic approach

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Abstract

Pancreatic cancer is ranked as the fourth leading cause of cancer-related mortality and is predicted to become the second leading cause of cancer-related death by 2030. The cause of this high mortality rate is due to pancreatic ductal adenocarcinoma's rapid progression and metastasis, and development of drug resistance. Today, cancer immunotherapy is becoming a strong candidate to not only treat various cancers but also to combat against chemoresistance. Studies have suggested that complement system pathways play an important role in cancer progression and chemoresistance, especially in pancreatic cancer. A recent report also suggested that several signaling pathways play an important role in causing chemoresistance in pancreatic cancer, major ones including nuclear factor kappa B, signal transducer and activator of transcription 3, c-mesenchymal-epithelial transition factor, and phosphoinositide-3-kinase/protein kinase B. In addition, it has also been proven that the complement system has a very active role in establishing the tumor microenvironment, which would aid in promoting tumorigenesis, progression, metastasis, and recurrence. Interestingly, it has been shown that the downstream products of the complement system directly upregulate inflammatory mediators, which in turn activate these chemo-resistant pathways. Therefore, targeting complement pathways could be an innovative approach to combat against pancreatic cancer drugs resistance. In this review, we have discussed the role of complement system pathways in pancreatic cancer drug resistance and a special focus on the complement as a therapeutic target in

pancreatic cancer.

Keywords: Pancreatic cancer, complement system, immunotherapy, drug resistance, nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT3), c-mesenchymal-epithelial transition factor (C-MET), phosphoinositide-3-kinase/protein kinase B (PI3K/AKT)

INTRODUCTION

Pancreatic cancer is ranked as the fourth leading cause of cancer-related mortality and is predicted to become the second leading cause of cancer-related death by 2030[1]. About 90% of these tumors are pancreatic ductal adenocarcinomas (PDACs), tumors of the exocrine part of the pancreas. The cause of this high mortality rate is due to PDAC's rapid progression and metastasis, low rate of early detection, and development of drug resistance[1].

In the last ten years, gemcitabine has been the most common treatment option for advanced-stage pancreatic cancer. However, even with this treatment option, the 5-year survival rate is still incredibly low, only 10% according to the American Cancer Society[2]. Unfortunately, the survival rate is continuing to decrease due to increased incidences of chemoresistance in pancreatic cancer cells. Researchers have investigated to determine the mechanism of chemoresistance to improve the efficacy of gemcitabine and other chemotherapeutic drugs. It has been found that a multitude of signaling pathways play a role in causing chemoresistance, the major ones being nuclear factor kappa B (NF-κB), signal transducer and activator of transcription (STAT3), c-mesenchymal-epithelial transition factor (C-MET), and phosphoinositide-3-kinase/protein kinase B (PI3K/AKT)[1,3,4].

Researchers have investigated these different signaling pathways to find a target they could inhibit to prevent their activation and sensitize the cancer cells to the treatment. However, with multiple pathways and a surplus of stimuli causing the activation, it has proven difficult to successfully inhibit them all. It has been found that inflammation which is a known element of cancer progression, plays a significant role in the activation of these different signaling pathways[5].

A huge mediator of inflammation within cancer is the complement system which is a part of the body's innate immune response and has been shown to induce a series of inflammatory responses to help fight infection and other diseases[6,7]. It has been proven that the complement system has a very active role within cancer progression, especially within establishing the tumor microenvironment[8]. In addition, it has been shown that the downstream products of the complement system directly upregulate inflammatory mediators, which in turn activate these chemo-resistant pathways[7].

This review article summarizes recent advances related to the role of the complement system in pancreatic cancer chemo-resistant signaling pathways, and the opportunity of targeting the complement system as a novel treatment to overcome drug resistance in pancreatic cancer.

Overview of the complement system

The complement system is an important mechanism that links innate immunity to adaptive immunity and helps the body fight foreign pathogens and abnormal host cells[9]. The complement system consists of multiple plasma proteins that sequentially interact with one another to mount an attack on the foreign substance. These complement proteins are widely dispersed throughout the body fluids and tissues and remain dormant until an infection or an abnormality is recognized. At that point, they are locally activated

and will trigger a series of strong inflammatory events. Three different pathways can trigger the activation of the complement system: the classical pathway, alternative pathway, and lectin pathway. Though these pathways differ in response to the local stimuli that cause their activation, all of these pathways lead to the cleavage of complement protein C3 into C3a and C3b, and C5 into C5a and C5b. C3a and C5a are inflammatory mediators or anaphylatoxins, and they contribute to inflammation through stimulation of histamine release and activation of other immune cells such as macrophages, eosinophils, and neutrophils[10]. C3b aids in opsonization which helps with phagocytosis of pathogens, and C5b initiates the assembly of the membrane attack complex, which is an essential mechanism to eliminate bacteria resistant to phagocytosis. Though the complement system is an important arm of the immune system, insufficient stimulation and/or overstimulation can be deadly to the host and is associated with autoimmune problems, chronic inflammation, infections, and even cancer[9].

Complement system and cancer

The complement system has been regarded as a component of the innate immune response against invading pathogens and "non-self-cells". However, when a tumor or malignancy is identified, the levels of complement proteins in tissues and fluids appear to vary[8]. It has been shown that the expression of complement proteins is increased in malignant tumors[8]. Complement system activation in the tumor microenvironment has been shown to lead to enhanced tumorigenesis and progression[8].

It has been well established that an inflammatory process is vital for the development of tumors in humans[11]. It was recognized that cancers develop from "subthreshold neoplastic states" which are caused by carcinogens that elicit a somatic change to the cells, but these altered cells can stay in normal tissues indefinitely, but when the second source of stimulation such as irritation or inflammation arises, these cells start to manifest as cancerous[5].

Through numerous experiments, it has been established that activation of the complement system is a very important component of tumor-promoting inflammation[12]. The inflammatory response is caused by the complement system's anaphylatoxins which are a critical step in the progression of tumorigenesis and cancer[12]. It was shown that C3a or C5a, which are both anaphylatoxins, can cause inflammation in a variety of ways by releasing histamine, activating other leukocytes, and stimulating the production of inflammatory mediators like TNF-alpha, IL-6, IL-1β, and IL-1[13]. It has been shown that these anaphylatoxins generated during tumor-associated complement activation can reshape the tumor microenvironment and play a role in tumor cell proliferation, resistance to chemotherapy, and angiogenesis within pancreatic cancer[14]. C5a has been shown to upregulate angiogenic factors such as vascular endothelial growth factor (VEGF) in cancer cells which plays a huge role in the development of newly formed blood vessels allowing the tumor to grow. C3a and C5a also play a huge role in the activation of tumor-associated macrophages (TAMs). These macrophages are activated by the anaphylatoxins, just like neutrophils and other leukocytes, but TAMs play a huge role in shaping the tumor microenvironment. TAMs produce a large number of strong angiogenic and lymphangiogenic growth factors, cytokines, and proteases, all of which increase neoplastic progression[5]. TAMs also produce IL-10, which dampens the anti-tumor response of cytotoxic T cells. In addition, TAMs cause an increase of inflammatory cytokines, which help with the invasiveness of the cancer cells allowing them to invade more tissues and spread easily.

Bonavita et al. reported that mice deficient in complement C3 were safe against chemical carcinogenesis in mesenchymal and epithelial tissue due to reduced inflammation[15]. It was also shown that the long pentraxin *PTX3* gene is a very important negative regulator of inflammation and complement activation. Mice deficient in this gene had a much higher susceptibility to chemical carcinogenesis, an increase in the

number of TAM's, and an increase of inflammatory chemokines inside the tumor[12]. The prolonged inflammation caused by the complement system in a tumor microenvironment increases the risk of neoplasticism transformation by causing the accumulation of cytokines, chemokines, growth factors, and reactive oxygen species, all of which can fasten the creation of a tumor-supportive microenvironment[16]. Further, the complement system also plays a significant role in creating cancer's defense mechanisms, especially with drug resistance. The complement's anaphylatoxins and downstream products like the activated macrophages have specifically been identified in upregulating specific chemoresistance signaling pathways[13].

Drug resistance pathway overview in pancreatic cancer

Drug resistance is one of the many reasons why the mortality rate of pancreatic cancer is so high. Through multiple experiments, it was shown that more than 165 genes are related to drug resistance[17]. These genes are involved with antioxidant activity, regulation of the cell cycle, signal transduction, and apoptosis. The four major chemo-resistant signaling pathways are NF-κB, C-MET, STAT3, PI3K/AKT[18] [Figure 1].

Gemcitabine has been the standard treatment for advanced-stage pancreatic cancer since it inhibits cell proliferation and causes apoptosis of tumor cells through the activation of the adenosine monophosphate-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) pathway[17]. For gemcitabine to function properly, after its uptake into the cell, it must be phosphorylated by deoxycytidine kinase[1]. The transport, phosphorylation, and activation of gemcitabine are all governed by numerous enzymes and proteins. One protein, in particular, which is essential for the function of gemcitabine is nucleoside transporter protein such as Human Concentrative Nucleoside Transporter (hCNT1), which is responsible for taking the drug into the cell allowing for it to inhibit cellular growth and cause apoptosis[1]. Further, it has been demonstrated that chemo-resistant signaling pathways cause downregulation of these nucleoside transporters and inactivate deoxycytidine kinase which decreases the efficacy of gemcitabine. In addition, these signaling pathways not only inhibit the activation and transport of gemcitabine but also improve the tumor microenvironment to better protect itself from damage.

Furthermore, another major cause of resistance to gemcitabine comes from inactivity of the human equilibrative nucleoside transporter 1 (hENT-1). Gemcitabine is a pyrimidine analog, and it relies on membrane transporters for its uptake. That is why "The presence and activity of the nucleoside transporters were considered as possible important determinants of gemcitabine cytotoxicity and clinical efficacy"[19]. hENT-1 and hCNT-1 are the most efficient transporters, but hENT-1 is the most widely expressed in human tissues and is overexpressed in different tumor types. Without the facilitated transport function of hENT-1, the entrance of gemcitabine into the cell and its ability to cause an effect is markedly decreased. It was shown that inhibition of the facilitated, diffusion-mediated nucleoside transport by the dipyridamole derivative BIBW22BS, resulted in a 30-100-fold decrease in activity for gemcitabine in various human cancer cell lines[20]. Whereas, high hENT-1 levels were associated with prolonged disease-free status and overall survival in patients receiving gemcitabine adjuvant chemotherapy[19].

After the entrance into the cell, gemcitabine is metabolized to active gemcitabine diphosphate and triphosphate by the enzyme deoxycytidine kinase, which leads to the inhibition of ribonucleotide reductase subunits 1 and 2, whose expression is associated with gemcitabine resistance[21]. In addition, gemcitabine is capable of inducing apoptosis in cancer cells primarily with a pathway involving caspase-8 and a mitochondrial-dependent caspase-9. The cascade led by caspase-8 is involved in death receptor-mediated apoptosis, whereas caspase-9 was thought to mediate chemical-induced apoptosis through the formation of an apoptosome. It was further reported that unlike inhibition of caspase 9, inhibition of caspase-8

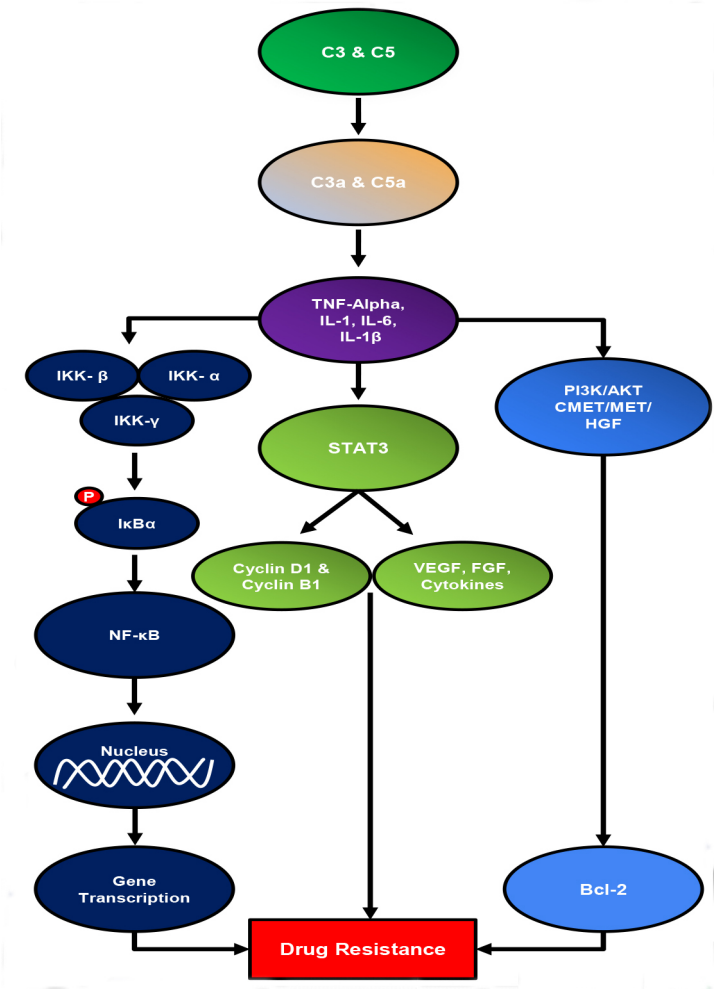

Figure 1. Complement system causes drug resistance through multiple signaling pathways in pancreatic cancer.

significantly decreases apoptosis. Furthermore, stable overexpression of the caspase-9 inhibitors, caspase-9S and X-linked inhibitor of apoptosis (XIAP) in the NSCLC cell line H460 failed to suppress apoptosis induced by various chemotherapeutic drugs; however, drug-induced apoptosis was blocked in H460 transfectants that expressed known inhibitors of caspase-8[22]. Researchers have studied the following signaling pathways as a way to better combat the chemoresistance that they cause to improve the survival rates of patients afflicted with pancreatic cancer.

NF-κB and complement system and its role in pancreatic cancer

Nuclear factor kappa B (NF-κB) has been shown to cause cancer progression, angiogenesis, and drug resistance in cancer, especially pancreatic cancer[4,23]. Ease of invasion and metastasis is one of the reasons which make the fatality rate of pancreatic cancer so high. It was shown that NF-κB was constitutively activated in about 67% of pancreatic adenocarcinoma but not in normal pancreatic tissue[24,25]. It has been previously reported that pancreatic cancer causes an upregulation of complement proteins, which in turn create more anaphylatoxins[26]. NF-κB is a transcription factor that can be activated through a classical signaling pathway or an alternative signaling pathway. The classical/canonical signaling pathway involves TNF-alpha, pro-inflammatory cytokines, and IL-1β, which will lead to the activation of the IKK-β. Then phosphorylates Iκβα, which will then cause the NF-κB dimer to be translocated into the nucleus so that it

can activate gene transcription[27]. It was previously shown that the anaphylatoxins C3a and C5a upregulate inflammatory mediators and cytokines, and cause direct stimulation of TNF-alpha and IL-1β[9]. In addition, these anaphylatoxins cause more macrophages to be recruited, which have also been shown to play a huge role in inflammation[13]. The complement system activates NF-κB through more than just direct inflammatory mediators. The extracellular signal-regulated kinase (ERK), which is a member of the MAPK family of signaling molecules, is shown to be activated by inflammatory cytokines which are stimulated by the anaphylatoxins such as IL-6 and TNF-alpha[28]. Following this activation, ERK causes changes in the tumor microenvironment by producing downstream effectors such as Ribosomal S6 Kinase (RSK). RSKs have been shown to change cell proliferation and survival through the activation of NF-κB[29].

Once NF-κB is activated, translocated into the nucleus, and able to affect gene expression which causes a variety of pro-tumor effects. NF-κB can cause the secretion of CXCL14 which is a chemokine that not only promotes angiogenesis and tumor growth but also plays a role in translocating NF-κB into the nucleus[27] [Figure 1]. With CXCL14 and the complement system synergistically activating NF-κB, the possibility for chemoresistance rises significantly. Gastrin, a normal digestive hormone has very high levels in pancreatic cancer cells but low levels in normal tissue and is involved with metastasis through altering the expression of ABCG2 which is known as the breast cancer resistance protein through the actions of NF-κB[4]. However, invasion and metastasis are only a few of the many negative effects caused by the activation of this transcription factor, the most harmful being chemoresistance. Gemcitabine has been noted as the first line of defense for pancreatic cancer, but its efficacy is being decreased by the chemoresistance caused by NF-κB, which prevents improvements in survival rates and prognosis. NF-κB through a signaling pathway can cause inhibition of human concentrative nucleoside transporter 1 and 3 and human equilibrative nucleoside transporters necessary for the uptake of gemcitabine into a tumor cell[30]. Without the ability to properly uptake the gemcitabine, the tumor cells will continue to survive and grow, decreasing the survival rate at a steadfast rate. It was also shown that NF-κB can induce drug resistance through the upregulation of the *MDR1* gene in cancer cells. The *MDR1* gene encodes for an integral membrane protein, P glycoprotein, which has a specific role in drug efflux, which is the ability of the cell to regulate its internal environment and remove harmful toxins or metabolites[31]. This is how the cancer cells can protect themselves from chemotherapeutic treatments. It was shown that inhibiting NF-κB would downregulate this gene and the P glycoprotein which would sensitize the cells to apoptosis. NF-κB has a multitude of pro-cancerous effectors, and it has been demonstrated that the complement pathway and its downstream effectors play a role in its activation.

Complement system and C-MET/MET/ hepatocyte growth factor pathway and its role in pancreatic cancer

C-MET is the mesenchymal-epithelial transition factor gene encoded for membrane-bound receptor tyrosine kinase RTKs. They are expressed by epithelial cells such as the liver, pancreas, prostate, and kidney, and this receptor binds with a ligand hepatocyte growth factor (HGF). Afterwards it activates a wide range of different signaling pathways, one being chemoresistance and tumorigenesis[32]. There is an aberrant activation of C-MET in tumors exhibiting malignant properties. Pancreatic cancer cells are one of the most malignant neoplasms across the world, so C-MET activation would be highly expressed in this cancer[33]. It has been shown that C-MET expression levels are 5-7 times higher in individuals with PDAC than with normal pancreatic tissue[34]. Interestingly, aberrant activation of C-MET can be caused by inflammatory cytokines[32]. The complement anaphylatoxins can cause this aberrant activation and initiate the signaling cascade that leads to chemoresistance and other pro-tumor effects [Figure 1].

HGF-MET signaling has been shown to encourage angiogenesis in cancer by inducing VEGF expression. In addition, HGF-MET stimulation induces apoptosis protection for non-self-lung cancer cells by down-regulating apoptosis-inducing factors[35]. It has also been shown that over-stimulation of C-MET causes non-self-cell lung cancer resistance to an EGFR inhibitor. Further, the HGF-MET pathway supports resistance through the PI3K/AKT signaling pathway. It has been shown that C-MET deletion in pancreatic neoplasia enhanced chemosensitivity to gemcitabine. The complement systems downstream inflammatory mediators activate the C-MET pathway, which causes chemoresistance through cross-talk with other signaling pathways such as PI3K/AKT.

Complement system and STAT3 and its role in pancreatic cancer

STAT3 play a major role in many signaling pathways and have been proven to play a role in promoting pancreatic cancer progression[36] [Figure 1]. STAT3 activation is dependent on the phosphorylation of tyrosine residue, Tyr705, which then allows dimerization of STAT3 allowing it to translocate into the nucleus and affect gene transcription[36]. Growth factors and oncogene proteins like Src and RAS have the ability to induce the phosphorylation of the Tyrosine residue and cause STAT3 activation. However, it was shown that inflammatory cytokines such as IL-6 and TNF-alpha also have the ability to cause activation of STAT3[37]. It has also been reported that these inflammatory cytokines are upregulated by the anaphylatoxins which have been shown to be in higher levels within pancreatic cancer patients. The anaphylatoxins produced by the complement system have proven to play a pivotal role in inflammation and cancer progression, and its downstream effectors, like Interleukin 6, cause the constitutive activation of STAT3[36]. It has also been shown that C3 complement overexpression showed a clear increase in STAT3 expression in gastric cancer cell lines[38].

Once the STAT3 signaling cascade has been activated, it can affect multiple aspects of cancer progression. For example, through the expression of angiogenic growth factors such as VEGF and FGF, angiogenesis of the tumor cell can be upregulated, which increases the ability to create its blood vessels and fasten the growth process. In addition, apoptosis can be inhibited through the upregulation of Cyclin D1 and Cyclin B1, which are regulatory proteins that have been implicated in tumorigenesis and the development of malignancy[39]. In concert with the complement system, STAT3 can upregulate inflammatory cytokines to increase inflammation and immune evasion. STAT3 can also enable the inhibitory functions of Treg cells[37]. Treg or T regulatory cells are responsible for maintaining peripheral tolerance and regulating the immune response. Now, if their inhibitory functions are upregulating, this in turn, causes an increase in the immunosuppressive environment in which cancer cells thrive. Finally, STAT3 plays a major role in energy metabolism for cancer cells. Cancer cells need the energy to replicate, so STAT3 upregulates glycolysis to make more ATP[40]. It has been shown that the energy cancer cells utilize, nearly 50%, comes from glycolysis, so the upregulation is a crucial prerequisite to tumor growth and cancer progression[40]. Finally, STAT3 plays an important role in chemoresistance in cancer cells. Further, STAT3 alters ATP-binding cassette membrane transporters, which are crucial to the process of drug uptake into the cell. If the drug is not able to breach the cell, then the therapeutic effect cannot be achieved[41]. Therefore, all the above reports suggest that STAT3 has been proven to enhance cancer progression and chemoresistance, and its phosphorylation can be initiated by the complement pathway's anaphylatoxins.

Complement system and PI3K/AKT and its role in pancreatic cancer

PI3K is a lipid kinase that regulates multiple cellular processes with its downstream effector, AKT. The PI3K/AKT signaling pathway has been considered a significant cause of chemoresistance in cancer therapy, inhibition of apoptosis, stimulation of cell growth, and modulation of cellular metabolism[42] [Figure 1]. This signaling pathway causes multi-drug resistance through interactions with ABC transporters, NF-κB, and others.

The PI3K/AKT pathway is activated by the production of 3-phosphorylated phosphoinositide which initiates the signaling cascade. However, it was shown that C3a binding to the C3a receptor leads to transduction of intracellular signals through various G-proteins and phosphorylation of the PI3K/AKT pathway, which causes chemokine synthesis in humans. Furthermore, C3a and C5a binding to their receptors cause an influx of calcium which initiates signaling cascades such as MAPK and AKT pathways[10,13]. Not only does the complement pathway use the PI3K/AKT pathway to help with the formation of their chemokines, but the binding of anaphylatoxins to their respective receptors also causes the activation of this pathway. In addition, IL-6, which is released by the anaphylatoxins, is required for pancreatic cancer progression through the activation of MAPK and PI3K/AKT signaling pathway[43]. One of the ways to activate the PI3K/AKT pathway is through the activation of the mutated *Kras* gene synergistically acting with IL-6. *Kras* mutation is shown to be the most commonly mutated gene in pancreatic cancer and drives the formation of pancreatic intraepithelial neoplasia[43]. It was also shown that without IL-6, cancer progression slows even in the presence of the *Kras* mutation[43].

Once the PI3K/AKT pathway has been activated, numerous downstream effectors cause cancer progression. For example, abnormal activation of the PI3K/AKT signaling pathway contributes to the upregulation of Bcl-2 expression, which causes cell survival by inhibiting the release of cytochrome c from the mitochondria[42]. This type of apoptosis prevention only adds to the multi-drug resistance already previously seen. Further, another downstream effector seen in many cancers is XIAP which has played a role in chemoresistance by inhibiting autophagy-induced apoptosis. It was shown that abnormal activation of PI3K/AKT induces XIAP expression[42]. Especially in pancreatic cancer cells, there is a cross-talk and activation between PI3K/AKT and NF-κB which results in multidrug resistance through the stimulation of Cyclin D1 to accelerate cell progression and tumor growth leading to drug resistance. Finally, pancreatic ductal preneoplastic lesions come from the differentiation of acinar cells to ductal cells, and the process of this occurring is called acinar to ductal metaplasia (ADM). This process is accelerated by mutated *Kras* and inflammation. However, p110-alpha, which is a catalytic subunit of PI3K-α can cause ADM in the presence or absence of pancreatic inflammation[44].

The complement-induced PI3K/AKT pathway not only acts with the *Kras* mutation to fasten the cancer progression, but also with NF-κB and other mediators to promote cell survival and chemoresistance.

CONCLUSION

The complement pathway is a part of the body's innate immune response capable of attacking pathogens and foreign abnormalities. However, with cancer, the complement pathway does more harm than good. It allows for increased inflammation through the release of anaphylatoxins, the activation of different signaling cascades through the induction of inflammatory mediators, and finally a nourishing tumor microenvironment. The most detrimental side effect of the complement system's activation would be the chemoresistance caused by the anaphylatoxins.

Pancreatic cancer has had one of the highest cancer-related mortality rates due to its fast metastasis and chemoresistance. The major signaling pathways discussed in this review article discussed have all been targets of therapeutic treatment in order to combat chemoresistance. Though these treatments have been shown to cause higher sensitivity to gemcitabine and other chemotherapeutic drugs, the problem has not been solved. The fact of the matter is that all of these signaling pathways are activated by different stimuli and can a cross-talk, therefore, inhibiting each one yields a futile response. However, it has been shown that targeting the complement pathway has been successful as a therapeutic treatment option for lung cancer in a few pre-clinical studies which indicate that inhibition of either C3a or C5a signaling inhibits cancer

progression in lung cancer models and other malignancies[45]. However, even with all the current evidence showing how the complement system upregulates inflammation and mediators that enable tumorigenesis, as of 2019, no clinical trials are targeting the complement system as a therapeutic approach. It has been shown that the complement system upregulates these signaling cascades, and targeting each main pathway has proven to be ineffective. If the complement's anaphylatoxins were targeted and the inflammatory response was removed, then there would be fewer inflammatory mediators upregulating these pathways. This could yield a more successful chemotherapeutic treatment without the obstacle of chemoresistance.

DECLARATIONS

Authors' contributions

Conceptualization: Pramanik KC, Hussain N
Original draft writing: Hussain N, Das D, Pramanik KC
Review and editing: Pramanik A, Pandey MK, Joshi V, Pramanik KC
Visualization: Pramanik KC
Supervision: Pramanik KC
Funding acquisition: Pramanik KC

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not Applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Immune checkpoint inhibitor resistance in soft tissue sarcoma

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Abstract

The emergence of immunotherapy as a cancer therapy has dramatically changed the treatment paradigm of systemic cancer therapy. There have been several trials evaluating immune checkpoint blockade (ICI) in soft tissue sarcoma. While there is generally a limited response in sarcoma, a subset of patients has durable responses to immunotherapy. This is attributable to a variety of factors including histologic subtype, tumor-infiltrating lymphocytes, and the tumor microenvironment among others. There is ongoing translational and clinical research evaluating ICI resistance in sarcoma and identifying therapeutic strategies to overcome this resistance. Herein, we provide a review of the current data, proposed mechanisms of resistance, and potential approaches to overcome this resistance.

Keywords: Sarcoma, soft tissue sarcoma, checkpoint inhibitor, immunotherapy, resistance

INTRODUCTION

Soft tissue sarcomas (STS) are a heterogenous group of cancers of mesenchymal origin[1]. They represent 1% of cancer diagnoses in the United States, with 13,130 diagnosed in 2020[2]. The prognosis of soft tissue sarcoma is poor, with up to 50% of patients with localized disease developing metastases and a median

survival of 12-24 months in the advanced setting[3,4]. There are more than 50 histologic subtypes of soft tissue sarcoma, each with a distinct clinical profile, prognosis, and response to treatment.

Sarcomas are generally divided into two categories based on genetics, simple and complex. Many simple subtypes are translocation driven and have limited neoantigens, such as synovial sarcoma (SS) and myxoid/round cell liposarcoma[5]. Complex sarcomas have more complex genetics, with potentially mutated protein targets for T-cells[5]. The most common of these include Undifferentiated Pleomorphic Sarcoma (UPS) and Leiomyosarcoma (LMS), and they have been found to have higher gene expression levels related to antigen presentation[5,6].

First-line therapy consists of anthracycline-based cytotoxic chemotherapy, and Doxorubicin remains the most active single agent with a response rate of up to 20-25%[7-11]. There have been attempts to improve this treatment approach with dose intensification and combination therapies, but these have had a limited impact on overall survival[4,8]. With the emergence and success of immunotherapy in other cancer subtypes, there has been interest in using this modality to treat soft tissue sarcomas[6]. While there are several modalities of immunotherapy, including T-cell transfer therapy, monoclonal antibodies, cancer vaccines, immune system modulatory and ICI, our review will focus on ICI[12]. This has led to multiple clinical trials of ICI and ICI combination therapy in advanced soft tissue sarcoma [Table 1].

An early phase II study evaluated the CTLA-4 inhibitor, ipilimumab, in advanced synovial sarcoma given their high expression of cancer testes antigen NY-ESO-1. Patients were treated with ipilimumab 3 mg/kg intravenously every 3 weeks for three cycles and then restaged. Six patients were enrolled and received 1-3 cycles of ipilimumab. All patients showed clinical or radiological evidence of disease progression after no more than three cycles of therapy. This study was stopped due to slow accrual and lack of activity[20].

SARC028 was a multicenter, two-cohort, single-arm, open-label phase II study evaluating the anti-PD-1 antibody, pembrolizumab, in 40 patients with advanced soft tissue sarcoma[13]. Patients received 200mg pembrolizumab IV every 3 weeks until progression or unacceptable toxicity. Histologies enrolled included UPS, dedifferentiated liposarcoma (DDLPS), SS, LMS. 18% (7/40, 95%CI: 7-33) of patients with soft tissue sarcoma achieved an objective response. This is clinically meaningful, although the prespecified objective response rate to meet their endpoint was 8/40 responses. The majority of these responses were in patients with UPS or DDLPS, and one patient with UPS achieved a confirmed complete response. The 12-week PFS in the soft tissue sarcoma cohort was 55%. The median duration of response was 49 weeks and overall survival (OS) was not reached in patients with UPS. In this study, adequate tumor biopsies were obtained from 81% of patients during treatment and were analyzed for pre-treatment PD-L1. PD-L1 was positive at the 1% threshold in only three samples, all of which were from patients with UPS. Two of the three were evaluable for response, and one had a complete response and the other had a partial response[13].

There was also a single center, phase II study evaluating nivolumab, an anti-PD-1 antibody, in twelve patients with advanced uterine leiomyosarcoma[21]. Patients received 3 mg/kg every 2 weeks intravenously of nivolumab until progression or unacceptable toxicity. There were no responses noted in this cohort, and the second stage was not opened due to lack of benefit[21].

The Alliance A091401 trial was an open-label, non-comparative, randomized, phase II study of nivolumab with or without ipilimumab, an anti-CTLA-4 antibody, in metastatic or locally advanced sarcoma[14]. Patients received intravenous nivolumab 3 mg/kg every 2 weeks or nivolumab 3mg/kg plus ipilimumab 1mg/kg every three weeks for four doses followed by nivolumab 3 mg/kg every two weeks for up to two

Table 1. Studies of checkpoint inhibition in sarcoma

Authors	Year	Study Type	Treatment	*N*	ORR	mOS	PFS
Tawbi HA, *et al.*[13]	2017	Phase II	Pembrolizumab	N = 80	18% (7/40 STS)	12.25m (8.50-18.25)	4.5m (2-5.25)
D'Angelo SP, *et al.*[14]	2018	Phase II	Nivolumab	*N*= 38	5% (2/38)	10.7m (5.5-15.4)	1.7m (1.4-4.3)
D'Angelo SP, *et al.*[14]	2018	Phase II	Nivolumab plus Ipilimumab	*N* = 38	16% (6/38)	14.3m (9.6-NR)	4.1m (2.6-4.7)
Wilky BA, *et al.*[15]	2019	Phase II	Axitinib plus Pembrolizumab	*N* = 33	25% (8/32)	18.7m (12-NR)	4.7m (2-9.4)
Martin-Broto J, *et al.*[16]	2020	Phase Ib/II	Sunitinib plus Nivolumab	*N* = 68	21% (12/58)	24m	5.6m (3.0-8.1)
Pollack S, *et al.*[17]	2020	Phase I/II	Pembrolizumab plus Doxorubicin	*N* = 37	19% (7/37)	27.6m (18.7-NR)	8.1m (7.6-10.8)
Kelly CM, *et al.*[18]	2020	Phase II	Talimogene laherpavec plus pembrolizumab	*N* = 20	35% (7/20)	18.68m (12.25-NR)	4.28m (3.15-NR)
Gordon, *et al.*[19]	2020	Phase II	Ipilimumab, Nivolumab, Trabectedin	*N* = 41	19.5% (8/41)	> 12.5m	> 6.0m

years. The most common sarcoma subtypes across both groups were leiomyosarcoma (34%), liposarcoma (6%), spindle cell sarcoma (13%), undifferentiated pleomorphic sarcoma (13%) and bone sarcoma (11%). In the nivolumab group (38 patients), the response rate was 5% (2/38, 92%CI: 1-16) and thus, did not meet its primary endpoint of objective response rate. The combination group (42 patients) had an overall response rate of 16% (6/38, 92%CI: 7-30) with a median PFS of 4.1 mo and OS 14.3 mo[14].

The results of clinical trials using checkpoint inhibition in soft tissue sarcoma have been varied, and there is an ongoing study into which sarcoma subtypes are more responsive to checkpoint inhibitors and if there are predictive biomarkers that can be used. This remains a challenge given the heterogeneity of different sarcoma subtypes and the rarity of each disease. Unlike other cancer types, biomarkers such as tumor mutational burden and PD-L1 expression have failed to identify good responders in soft tissue sarcoma. Although a small proportion of patients do respond to checkpoint inhibitors, the majority do not, likely due to primary resistance to checkpoint inhibition. In those who do respond, most will progress, which is likely due to acquired resistance.

Herein, we will highlight here mechanisms of resistance, research evaluating predictive biomarkers in STS and current approaches to overcome resistance to checkpoint inhibition.

RESISTANCE MECHANISMS

Unlike other more immunogenic cancer types such as melanoma, only a minority of patients with soft tissue sarcoma develop durable clinical responses to ICI (primary resistance) and most of those who do respond will eventually progress (acquired resistance). In order for the immune system to respond effectively, cancer cell-specific antigens that are recognizable by antigen-presenting cells are required, T cells must be primed and activated with the ability to infiltrate tumors, and cancer cells must recognize and destroy cancer cells[22]. There also must be a balance of stimulatory and suppressive signaling that favors continued cytotoxicity by T-cells within the tumor microenvironment (TME)[15,22].

Tumors are generally thought of as immunogenic (or hot) or non-immunogenic (or cold). Sarcomas are generally characterized as non-immunogenic, cold tumors with limited immune cell infiltrate, low tumor mutational burden (TMB) and low PD-L1 expression, which is thought to contribute to their primary resistance to ICI[23,24]. This is by tumor intrinsic or extrinsic mechanisms. Tumor intrinsic mechanisms

include lack of antigenic proteins, lack of antigen presentation, genetic T-cell exclusion or insensibility to T-cells[25,26]. Oncogenic signaling pathways also contribute to tumor intrinsic resistance to ICI[26]. Tumor extrinsic mechanisms include the absence of T cells, inhibitory immune checkpoints and immunosuppressive cells including tumor-associated macrophages (TAMs) and T-regulatory cells (Tregs)[25]. Upregulation of Tregs can induce immunologic tolerance and an increase in M2 TAMs suppress the TME and correlate with progression[12]. Within STS, there is wide heterogeneity, with response rates that are variable among histologies. Prior studies have shown an increase in sensitivity to ICI among such histologies as ASPS, UPS and DDLPS[14,27,28].

Given that T cell responses are central to the efficacy of ICI, there have been several studies evaluating tumor infiltrating lymphocytes (TILs) within sarcomas prior to and on treatment. D'Angelo[29] and colleagues evaluated TILs in 50 sarcoma specimens to further evaluate the immune milieu. They noted infiltration of TILs and TAMs in 98% and 90% of tumors, respectively. They evaluated subsets of TILs with the median number of each subset and noted CD3+ cells 3.3% (0%-33.2%), CD8+cells 1.2% (0%-14%), CD4+ 0.2 (0%-13.6%), and FOXP3+ 0.1 (0-3.6%)[29]. There was an increased number of CD8+ cells in larger tumors or those who presented with metastatic disease, which is potentially indicative of T-cell exhaustion[29]. This study found that low CD3+ and CD4+ correlated with better survival, although contrary findings have been noted in other studies. In a cohort of 128 high-grade STS, increased density of CD8+ and CD3+ TIL infiltrates were associated with favorable OS, DSS and DFS when compared to low-density CD8+ and CD3+ infiltrates[30]. SARC028 correlative analysis evaluated changes in tumor-associated immune infiltrates from baseline to early on-treatment biopsies. They found that both effector memory cytotoxic T-cells and Tregs were subsequently increased after PD-1 blockade (P = 0.054)[28]. Analyses also showed that higher Treg percentages and higher density of cytotoxic T-cell infiltrates at baseline had longer median PFS[28]. A retrospective study of 81 patients with liposarcoma, LMS, UPS, and SS found that more highly mutated STS subtypes expressed higher levels of genes related to antigen expression and T-cell infiltration and inflammation[5]. Higher expression was seen in UPS and LMS compared to SS. They also had higher levels of *CD3D*, a marker for T cell infiltration, and *CD8A*, a marker for CD8+ T-cells[5].

PD-L1 expression in sarcoma is varied, and the data regarding the correlation between PD-L1 expression and responsiveness to ICI in STS is variable, and at this time there is not a consistent correlation[5,29,30]. In a cohort of resected UPS, SS, AS, ASPS, and ES, any level of PD-L1 expression in tumor cells was positive in 28.1% (n = 36/128) of cases. The highest level was found in UPS and the patients with UPS who were PD-L1 positive were noted to have better OS and PFS compared to those patients who were PD-L1 negative[30]. An analysis of 50 sarcoma specimens noted tumor cell PD-L1 expression of 12% and lymphocyte and macrophagic PD-L1 expression in 30% and 58% of specimens respectively with no correlation with prognostic indicators[29]. Pollack and colleagues evaluated PD-1 expression by immunohistochemistry (IHC) in common STS subtypes and scored them from 0 to ≥ 5. 35% (n = 28) of tumors had PD-L1 expression, and 51% (n = 41) had PD-1 expression of ≥ 2, with UPS associated with higher expression levels of both PD-L1 and PD-1. Although this study was not designed to evaluate survival outcomes, no correlation was found[5]. A pooled analysis of sarcoma clinical trials evaluated PD-L1 expression (≥ 1%) in soft tissue sarcoma. This was observed in 13.6% (n = 21/156) of patients with available data, and in this group, there was a corresponding ORR of 30% in PD-L1 positive tumors. In the PD-L1 negative tumors, the response rate was 7%[31]. Correlative analysis from the SARC028 trial noted PD-L1 positivity in 5% of tumors. Both of the PD-L1 positive tumors were UPS and responded to pembrolizumab, although five other patients responded and were PD-L1 negative[28]. Given the varied expression of PD-L1, particularly between different histologic subtypes, the prognostic and predictive significance of PD-L1 expression remains indeterminate[12].

The sarcoma TME varies widely by histology and can influence outcomes in patients with sarcoma[23]. Within the TME, immunosuppressive cytokines, abnormal perfusion from tumor angiogenic networks and metabolic conditions can inhibit T-cell infiltration and function. LMS have poor responses to ICI, and previous studies have shown poor T-cell infiltration in these tumors[29,31]. Gene expression profiling has also revealed high-level expression of macrophage-associated genes, such as CD 163 and CD68, which was associated with worse disease-specific survival in nongynecologic LMS[32]. Petitperez and colleagues studied TME gene expression profiles within STS based on immune classifications from immune low to immune high and highly vascularized[33]. They found most LMS classified to the low immune classes (classes A&B), DDLPS in the highly vascularized group (class C) and immune high (classes D&E) distributed across a variety of histologies[33]. The immune high group (E) is characterized by tertiary lymphoid structures rich in B-cells. Despite high or low CD8+ cell density, the presence of B-cells was the strongest prognostic factor with improved survival and high response to pembrolizumab therapy.

In those patients who initially respond to ICI, a proportion will eventually progress after ICI. Many of the mechanisms are similar to de novo resistance, but much remains unknown regarding the mechanism behind acquired resistance within sarcoma. There are several potential mechanisms including downregulation of tumor antigen presentation and subsequent lack of T-cell recognition, loss of T-cell function and development of escape mutation variants[25]. The immunoediting hypothesis refers to the interactions between the immune system and tumor cells that eventually lead to the inability of the immune system to recognize the tumor[34,35]. Anagnostou and colleagues matched pre-treatment and ICI resistant non-small cell lung cancer and found that resistant tumors had a loss of 7-18 putative neoantigens, many of which generated peptides responsible for host immune response[36]. There have been studies in melanoma regarding acquired resistance to PD-1 blockade. Zaretsky and colleagues performed whole-exome sequencing on the paired baseline and relapsing biopsy samples in four patients with melanoma who had initially responded to pembrolizumab therapy[37]. Two of the four patients revealed loss-of-function mutations in genes encoding interferon-receptor-associated Janus Kinase 1(JAK1) or JAK2. This resulted in insensitivity to the antiproliferative effect of interferon on cancer cells. They also noted mutations in beta-2-microglobulin which led to the loss of major histocompatibility complex class 1 surface expression[37].

BIOMARKERS OF RESPONSE

A focus of sarcoma research has been on predictive biomarkers which may delineate those who are likely to respond to ICI [Table 2], although predictive biomarkers remain elusive, and to date, there are no clearly defined biomarkers for soft tissue sarcoma.

Prior studies have noted that a high baseline neutrophil-to-lymphocyte ratio was associated with aworse prognosis in sarcoma[27,38,39]. The increased neutrophil-to-lymphocyte ratio has also been associated with inferior PFS in sarcoma patients who were treated with axitinib and pembrolizumab, but further elucidation of whether this is specifically predictive in the setting of ICI[27]. Sarcoma patients who were treated with axitinib and pembrolizumab were also noted to have improved outcomes if they had higher plasma angiogenic activity at baseline[27]. There is still further investigation to elucidate the prognostic implications of this finding.

There is interest in the emergence of DNA methylation profiles as predictive biomarkers in sarcoma patients, particularly those treated with ICI. DNA methylation has been implicated in tumorigenesis in a variety of tumors including sarcoma. A recent retrospective analysis of 35 recurrent sarcoma patients who were treated with anti-PD-1 ICI, most of which were treated with Pembrolizumab, noted DNA methylation differences between responders and nonresponders[23]. The most prominent pathway differences were seen

Table 2. ICI biomarkers in soft tissue sarcoma

PD-1/pd-l1 expression
cd8+ T-cells
Regulatory T cells
Tumor-associated macrophages
Tumor mutational burden
Neutrophil-to-lymphocyte ratio
DNA methylation profiles
Sarcoma Immune Class

in Rap 1 signaling, focal adhesion, adherens junction, pathways in cancer and extracellular matrix -receptor interaction[23]. In this study, PD-L1 expression and density of TIL subsets were evaluated and there was no correlation with response to ICI[23]. DNA methylation profiling was evaluated in 36 angiosarcoma specimens and revealed two subtypes (A and B) which were divided into four subclusters. Survival analysis showed better overall survival in cluster A at 22 months compared to cluster B at 6 months ($P = 0.046$)[40].

In other cancer types, there have been established biomarkers that predict response including tumor mutational burden and expression of the immune checkpoint molecules PD-1/PD-L1[26].

A retrospective study by Lu and colleagues of 18 metastatic sarcoma patients receiving anti-PD 1 therapy low TMB in all patients (range 1.12-3.45 mutations/MBs)[41]. Within sarcoma, PD-L1 positivity rates are low and have not been noted to be a consistent biomarker[28,29]. Data has been inconsistent with some studies noting improved survival in PD-L1 positive patients[31] and several without correlation between PD-L1 expression and outcomes[5,28]. A retrospective study of 18 metastatic sarcoma patients receiving anti-PD 1 therapy noted a PR in 22.2% (4/18) and SD in 50% (9/18) at 12 weeks with an ORR of 18.3% in soft tissue sarcomas. Whole exome sequencing was performed pre-treatment in 8 patients and did not note associations of PD-L1 expression with clinical response[41]. These studies highlight the unreliability of PD-L1 as a biomarker in STS.

Rates of TILs and TAMs have also been evaluated as potential biomarkers for response. Correlative analysis of SARC028 noted a higher percentage of tumor immune cell phenotypes in those patients who had responses to pembrolizumab[28].

Immunohistochemical staining can be used to confirm CD68 and CD163 positive macrophages. As noted above, a subset of non-gynecologic LMS has been noted to have dense infiltrates of these TAMs were found to have shorter disease-specific survival, although this was not seen in uterine LMS[32]. LMS has been noted to have high levels of T-cell-related gene expression, and it is postulated that TAMs are likely critical to immune invasion in these tumors given the poor clinical outcomes with single-agent ICI in LMS[5,21,32].

The TME has also recently been of interest as a prognostic indicator of response to ICI. STS biopsies from the SARC028 clinical trial were placed into their sarcoma immune classes and the ORR in group E was 50% ($n = 5/10$), followed by group D of 25% ($n = 3/12$) and group C of 22% (2/9)[33]. These are all higher than the ORR of 21.2% in the overall cohort. There were no responders in groups A and B. Group E also had improved PFS when compared to groups A &B ($P = 0.023$ and $P = 0.0069$, respectively)[33]. This study has laid the groundwork for potentially risk stratifying patients prior to treatment and identifying those who would be more likely to respond.

OVERCOMING RESISTANCE

Given the relatively low response rates to ICI in soft tissue sarcomas, there has been interest in manipulating the immune environment to increase responses [Figure 1]. Many sarcomas have limited neoantigens and therefore, limited immunogenicity without the generation of tumor-specific T-cells. There are several approaches that are combined with ICI to overcome this limitation including cytotoxic chemotherapy and oncolytic viruses. There is also interest in using drugs, such as tyrosine kinase inhibitors (TKIs) in combination with ICI to target the TME and overcome its suppressive influences which is mediated through immunosuppressive immune cells and cytokines.

Cytotoxic chemotherapy is an effective inducer of immunogenicity and increases inflammatory cytokines. There is evidence, using lung adenocarcinoma models, that chemotherapy can sensitize tumors to host antitumor T-cell immunity[42]. A lung adenocarcinoma mouse model was able to show that an antitumor CD8(+) T-cell response could be induced with immunogenic chemotherapy[42]. Within sarcoma, there have been several trials combining chemotherapy with immunotherapy to this end. The SAINT trial evaluated the combination of trabectedin (1.2 mg/m^2 IV q 3 weeks) with nivolumab (3 mg/mg q 2 weeks) and Ipilimumab (1 mg/m^2 q 12 weeks)[19]. Forty-one patients with previously untreated advanced or metastatic soft tissue sarcoma were included. The overall response rate was 19.5% with a disease control rate of 87.8%. Median PFS was > 6.0 months and median OS was > 12.5 months[19]. These data suggest synergy with trabectedin and Ipilimumab and Nivolumab. A phase 1/2 study by SM Pollack and colleagues evaluated the combination of doxorubicin (45 and 75 mg/m^2 q3 weeks) and pembrolizumab (200 mg q3 weeks) in 37 patients with advanced sarcoma who had not received prior anthracycline[17]. The ORR was 13% for phase 2 patients and 19% overall with a median PFS of 8.1 months (95%CI: 7.6-10.8) and median OS 27.6 mo (95%CI: 18.7-not reached). While this study did not reach its primary endpoint (ORR of 15% with 85% power), there was a clinically significant increase in PFS compared to historical studies. Two of three patients with UPS and two of four patients with dedifferentiated LPS had durable partial responses. In 29 patients, there was evaluate IHC for correlatives. 66% had a PD-L1 score of 0, and PD-L1 was not associated with PFS or OS. Tumor-infiltrating lymphocytes were present in 21% of evaluable tumors and associated with inferior PFS (p=0.03). They assessed serum cytokine levels before treatment and during the first two cycles. Granulocyte macrophage colony-stimulating factor levels increased each cycle, and IL-15 levels dropped following doxorubicin treatment. Circulating IL-2R, IP10, and CD30 levels rose sharply after cycle one and levels of IL-8 dropped[17].

Oncolytic viruses, engineered viral vectors that selectively infect and replicate within cancer cells, are also being combined with checkpoint inhibitors in the treatment of sarcomas[15]. The innate immune system is also able to recognize these viruses as foreign and initiate an immune response[43]. A recent phase II study evaluated the combination of intralesional talimogene laherparepvec (T-VEC, the first dose, ≤ 4 mL × 10^6 plaque-forming units [PFU]/mL; second and subsequent doses, ≤ 4 mL × 10^8 PFU/mL injected into palpable tumor site(s) on day 1 of each 21-day cycle) with pembrolizumab (200 mg/dose q 3 weeks) in 20 patients with locally advanced or metastatic sarcoma[18]. The best ORR at 24 weeks was 30 % (n = 6, 95%CI: 12%-54%) and overall was 35% (n=7, 95%CI: 15%-59%). Median PFS was 17.1 weeks (95%CI: 12.6-NR weeks) and median disease-specific survival was 74.7 weeks (3-sided 95%CI: 49.0-NR weeks). Two of the patients who responded to treatment had disease progression while on ICI prior to entering the study, which may suggest synergism between ICI and T-VEC. There were 11 patients with paired evaluable tumor samples and 55% (n = 6) converted from PD-L1 negative at baseline to PD-L1 positive after treatment. Six of the seven patients who responded had evaluable tissue, and in this cohort, there were one PD-L1 positive baseline tumor and four PD-L1 positive post-treatment tumors[18]. No patient tumors in the refractory group (n = 13) were PD-L1 positive at baseline and five were positive after treatment. They also evaluated

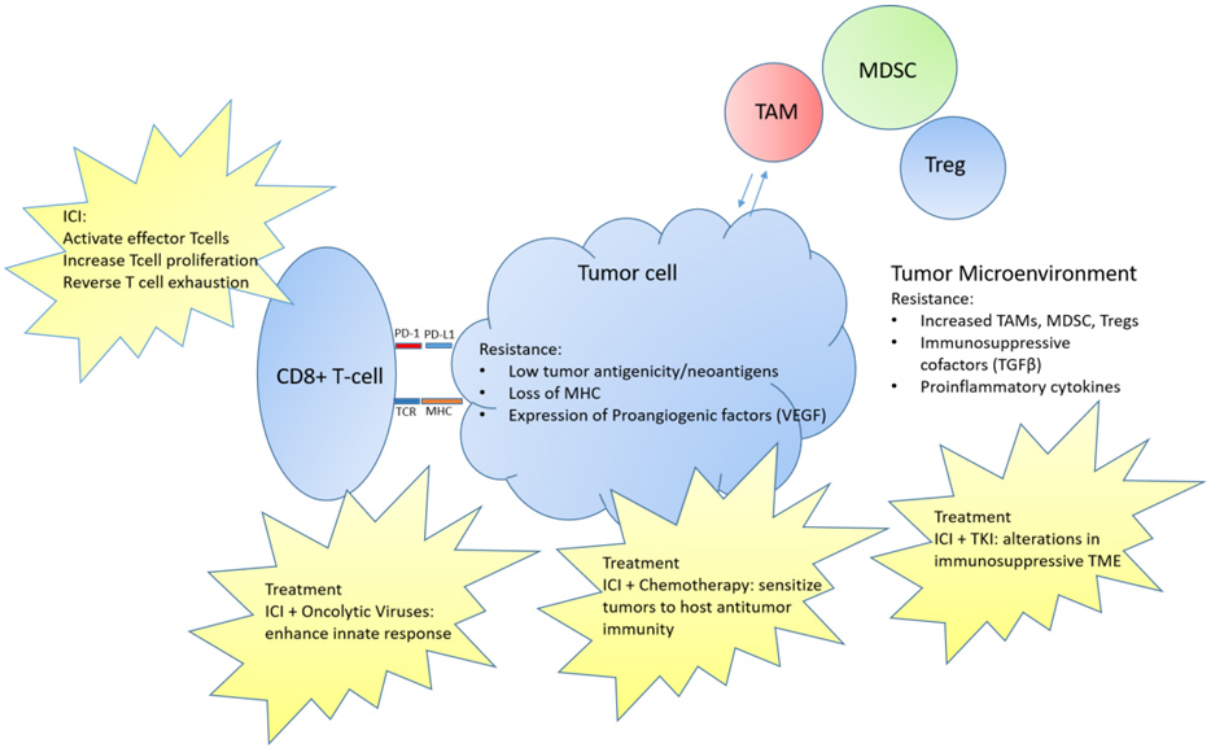

Figure 1. Resistance mechanisms to immunotherapy in soft tissue sarcoma with current treatment mechanisms aimed at overcoming resistance. ICI: Immune checkpoint inhibitor; MDSCs: myeloid-derived suppressor cells; MHC: major histocompatibility complex; PD-1: programmed cell death 1; TAMs: tumor-associated macrophages; TCR: T cell receptor; TKI: tyrosine kinase inhibitor; TME: tumor MIcroenvironment.

tumor-infiltrating lymphocyte (TIL) scores which were higher in the response groups (mean TIL score 3) compared to the unresponsive group (mean TIL score 2)[18]. Responsive patients also had the presence of aggregates of CD3+/CD8+ TILs in the tumor on the pre-treatment biopsy, particularly at the infiltrating edge, and this number increased in the post-therapy samples. Comparatively, there were minimal CD3+/CD8+ infiltrates in the nonresponsive patient tumors. This is a potentially promising therapy and additional investigation is ongoing in sarcoma.

There have been several studies evaluating TKIs in combination with ICI to overcome the immunosuppressive microenvironment. There are several well-known mediators of this environment including vascular endothelial growth factor (VEGF) and Transforming growth factor-ß. VEGF and other proangiogenic cofactors are necessary for tumor growth and spread. TKIs with activity against these factors have produced responses in metastatic sarcoma in prior studies[44-46]. An in vitro study of cocultures of sarcoma evaluated the role of TKIs and PD-1 based therapy[47]. In this study, human osteosarcoma and SS cell lines were treated with sunitinib. They were then cocultured with dendritic cells (DCs) and the phenotype of these DCs was determined by flow cytometry. Mature DCs were cultured with autologous T cells and the T cells were evaluated for PD-1 expression, proliferation, Treg induction, and IFN-γ production, before and after nivolumab exposure. They found that treatment with sunitinib induced upregulation of PD-L1 on sarcoma cells, induced maturation of DCs, and reduced Treg induction. There was no effect on T cell proliferation or T cell subpopulations. Treatment with nivolumab induced IFN-γ-producing effector T cells[47].

A phase II single-arm study by Wilky *et al.* combined axitinib (5mg twice daily), an oral TKI, with pembrolizumab (200mg/dose on day 8 and every 3 weeks for up to 2 years) in 33 patients with advanced sarcoma[27]. ORR was 25% (n = 8, 95%CI: 12.1-43.8) with clinical benefit rate of 53.1% (n = 17; 95%CI: 35.0-70.5). In the intention to treat analysis, median PFS was 4.7 months (95%CI: 3.0 to 9.4) and median OS was 18.7 months (95%CI: 12.0 to NR). In this study, 11 patients had alveolar soft part sarcoma (ASPS). ASPS is a rare translocation-driven sarcoma subtype that frequently presents in adolescents and young adults. There is a growing body of evidence that these tumors are responsive to both TKIs and ICI and several studies are ongoing[48]. In this study, the ORR in the ASPS cohort was 54.5% (95%CI: 24.6-81.9). The response rate in the ASPS was greater than that would be expected with either axitinib or pembrolizumab alone, and four of five patients who achieved a partial response had not achieved a partial response with at least on previous TKI. Correlatives and exploratory analyses are still underway.

A recent phase Ib/II trial evaluated the combination of nivolumab (3 mg/kg IV on day 15, then every 2 weeks) with sunitinib (37.5mg for the first 14 days, then 25mg per day) in 68 patients with advanced soft tissue sarcoma who had progressed on prior therapy[16]. The 6-month PFS was 48% (95%CI: 41-55%) with a median PFS of 5.6 months (3.0-8.1). The median overall survival was 24 months with an 18-month survival of 67% (95%CI: 59-74%). The ORR was 21%, with 100% of responding patients alive at 18 months. These response rates, PFS and OS are favorable compared with activity in anti-PD-1 or sunitinib monotherapy in previous trials[13,49].

These combination trials show promise in the quest to overcome resistance innate to many sarcomas. Further combination trials are underway.

CONCLUSION

Treatment of sarcomas remains difficult given the heterogeneity in immunogenic features of histologic subtypes and varied responses to ICI due to underlying primary or acquired resistance. There remains interest and promise in combining ICI and immunosensitizing agents to overcome underlying resistance mechanisms within sarcomas and the TME. Further, identifying reliable biomarkers to determine who responders to ICI will be remains an important but complex undertaking. Ongoing studies to better define the immunologic landscape, the immunosuppressive role of the TME and subsequent resistance mechanisms will improve understanding of this complex disease with the goal of improving clinical outcomes.

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Authors' Contributions

Conceptualization, writing-original draft: Eulo V
Conceptualization, writing-review and editing: Van Tine BA

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Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Commentary

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Natural bioactive compounds: a potential therapeutic strategy to sensitize bladder cancer to cisplatin treatment?

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Abstract

Bladder cancer (BC) is the tenth most common cancer, and its incidence is steadily rising worldwide, with the highest rates in developed countries. Neoadjuvant cisplatin-based chemotherapy followed by radical cystectomy is the standard therapy for patients with muscle-invasive bladder cancer. However, less than 50% of patients initially respond to this treatment and nearly all of them eventually develop resistance, which is an important barrier to long-term survival. Therefore, there is an urgent need to understand the mechanisms of cisplatin resistance in BC and develop ways to counteract them. Several preclinical studies have demonstrated that naturally derived bioactive compounds, such as phytochemicals and flavonoids, can enhance the antitumor activity of cisplatin, with minimal side effects, by targeting different pathways involved in cisplatin sensitivity and resistance. However, their poor bioavailability has been one of the main problems for their successful introduction into clinical practice. At present, however, many new formulations with greatly increased bioavailability are under study in several clinical trials with encouraging results.

Keywords: Bladder cancer, muscle-invasive bladder cancer, cisplatin, chemoresistance, natural products, curcumin, bioavailability

Bladder cancer (BC) is a current clinical problem due to its high incidence, prevalence, and mortality[1,2]. At diagnosis, 70% of patients present with non-muscle-invasive BC, a highly recurrent tumor treated by transurethral resection followed by BCG instillation. However, a high proportion of patients have recurrence and progress to muscle-invasive BC (MIBC), which is also present in 30% of patients at diagnosis[3]. At present, the treatment of MIBC has several limitations. Cisplatin-based neoadjuvant chemotherapy followed by radical cystectomy is the standard therapy, with the highest level of evidence, but it is only effective in 25%-50% of patients and confers a limited survival benefit[4]. Moreover, this survival benefit is only observed in patients with pathological response, while the delay in cystectomy in those who do not respond has a negative prognostic impact, highlighting the need for predictive biomarkers to identify those patients likely to benefit from cisplatin-based neoadjuvant treatment[5]. In addition, adjuvant chemotherapy has been recommended for patients with high-risk MIBC, although randomized trials have not provided conclusive evidence on the impact of this approach[6-8]. Furthermore, nearly 50% of patients will develop metastatic disease after undergoing radical cystectomy[3], and platinum-based chemotherapy is considered the cornerstone of treatment for these patients as well[9]. Nevertheless, immunotherapy - especially immune checkpoint inhibitors (ICIs) - are potentially effective in metastatic BC (mBC)[10,11], although only 20%-30% of mBC patients respond and there are no reliable predictive biomarkers of response[12]. Importantly, with the approval of ICIs for the treatment of mBC, they are now being studied in the neoadjuvant setting for MIBC with promising results[13]. Thus, based on this evidence, it is clear that cisplatin still plays an essential role in the treatment of MIBC. However, less than 50% of patients initially respond, and nearly all of them eventually develop resistance, which represents an important barrier to long-term survival.

In this issue of *Cancer Drug Resistance*, Rajendran *et al.*[14] elegantly reviewed the mechanisms of cisplatin activity and resistance in BC and the potential of bioactive natural compounds, such as phytochemicals and flavonoids, to overcome this resistance and improve therapeutic response. As they pointed out[14], resistance to cisplatin is a complex, multifactorial process that can be attributed to specific mechanisms intrinsic to BC biology or to general mechanisms common to different tumor types or drug pharmacokinetics. Several factors have been associated with resistance to cisplatin, including decreased intracellular drug concentration mediated by drug efflux pumps, alterations in DNA repair genes, and increased drug cytosolic inactivation[15]. I fully support their argument[14] that a greater understanding of the mechanisms of cisplatin activity and resistance, as well as the identification of potential biomarkers of response, is required so that we can select patients likely to respond to cisplatin and to improve strategies to fight resistance mechanisms. One such strategy is based on targeting the effectors and pathways involved in chemoresistance with natural compounds that can potentially synergize with platinum drugs, such as cisplatin. The antitumor effects of natural products are generally attributed to suppression of cell proliferation and metastasis, induction of apoptosis, and stimulation of autophagy, with minimal side effects[16].

Based on our group's experience in different tumor types, including mBC[17-19], we can highlight the potential and promising role of curcumin and its derivatives as chemosensitizing agents[20]. Curcumin (*diferuloylmethane*) is a hydrophobic polyphenol. It is the major curcuminoid in the spice turmeric (77% curcumin, 17% demethoxycurcumin, and 3% bis-demethoxycurcumin) and is derived from the rhizome of the herb *Curcuma longa*. Many of curcumin's antitumor properties have been attributed to its role as an anti-inflammatory and antioxidant or to its ability to modulate the cell cycle, several pathways, and transcription factors involved in proliferation, apoptosis, migration, invasion, angiogenesis, metastasis, and chemoresistance. Moreover, it has a very low toxicity profile in humans[20]. In particular, several studies have demonstrated that curcumin can enhance cisplatin sensitivity and prevent drug resistance in different

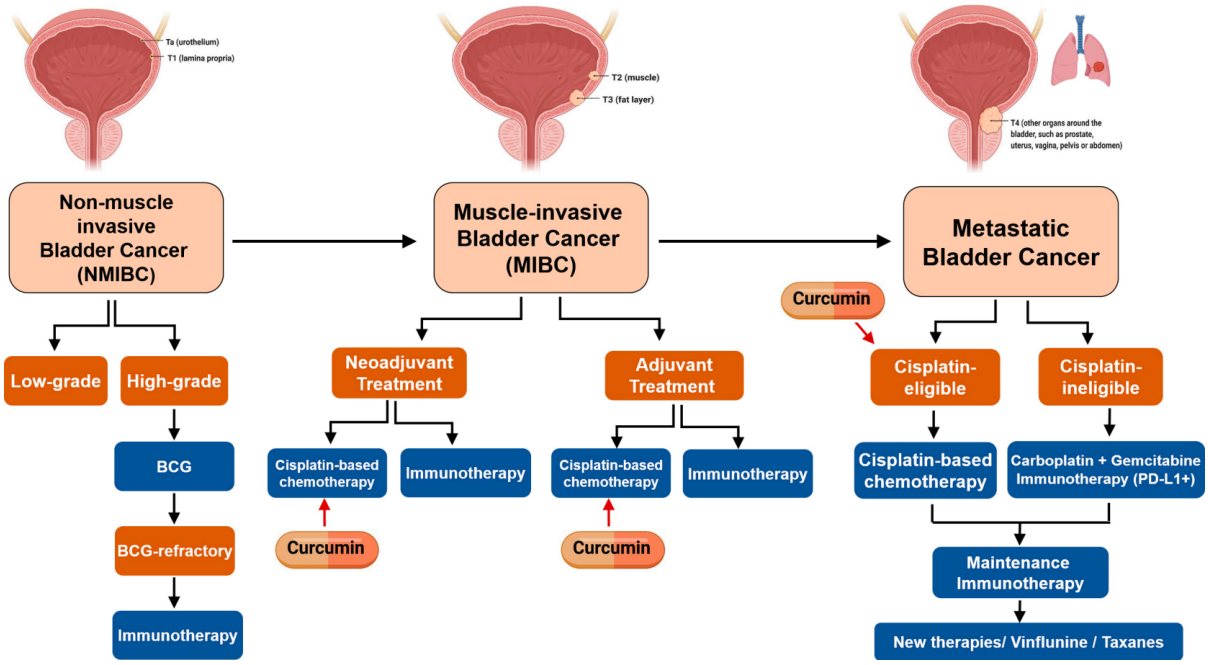

Figure 1. Potential clinical settings for the combined treatment of cisplatin plus curcumin in BC. Created with BioRender.com. BC: Bladder cancer.

tumor types[21]. Interestingly, Park *et al.*[21] demonstrated that co-treatment with curcumin and cisplatin synergistically induced apoptosis through ROS-mediated activation of the MEK/ERK pathway and the downregulation of survival proteins in BC cell lines and xenograft mouse models. More recently, it has been shown that the curcuminoid demethoxycurcumin enhanced the chemotherapeutic efficacy of cisplatin on HER2-overexpressing BC cells and inhibited the HER2-induced drug resistance of tumor cells through the inhibition of the PI3K/AKT signaling pathway[22]. Furthermore, it is well known that a major obstacle for the clinical use of cisplatin is its associated toxicities, including renal damage, deafness, and peripheral neuropathy, which negatively affect the overall efficacy of treatment[15]. Interestingly, curcumin not only increases the efficacy of cisplatin in several cancer models but also decreases cisplatin-related toxicity[16,23,24]. The potential clinical scenarios for the combined treatment of cisplatin plus curcumin in BC are depicted in Figure 1.

However, although it is true that *in vivo* and *in vitro* data are promising, the clinical application of these naturally derived bioactive compounds has been limited by their poor bioavailability in humans due to poor absorption and water solubility as well as to their rapid metabolism and systemic elimination[25,26]. Nevertheless, to solve this problem, in recent years, many efforts have been focused on obtaining new promising bioavailable formulations or delivery strategies: liposomes, micelles, phospholipid complexes, microemulsions, nano-emulsions, emulsions, solid lipid nanoparticles, nanostructured lipid carriers, biopolymer nanoparticles, and microgels[27,28]. Nonetheless, as proposed by Rajendran *et al.*[14], high quality pharmacokinetic and pharmacodynamic studies are required for proper dosing of these new compounds. Moreover, increasing our understanding of how these compounds work is critical to developing synthetic derivatives with improved pharmacokinetics and greater bioavailability and efficacy.

In conclusion, although cisplatin is a clinical mainstay for the treatment of MIBC, many tumors unfortunately develop resistance and are refractory to treatment. As Rajendran *et al.*[14] pointed out,

combination therapies using natural products represent a promising effective and novel strategy to overcome this resistance. For instance, the combination of curcumin and cisplatin could be a potential synergistic strategy to attenuate cisplatin-related adverse effects and decrease resistance. Finally, improved delivery strategies and new formulations offer encouraging ways to increase the bioavailability of these natural products. Hopefully, these strategies will be tested in further randomized, double-blind, placebo-controlled clinical trials in BC patients and incorporated into clinical practice. Nevertheless, although clinical trials using new bioavailable curcumin formulations are certainly mandatory to evaluate the optimal dosage, safety, and antitumor activity, based on our group's previous preclinical results[19] as well as on those presented by Rajendran *et al.*[14], I suggest that the combination of curcumin plus a platinum agent, such as cisplatin, could be an effective and reliable approach for the management of several cancers, including BC.

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Cancer Drug Resistance

Review

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Characteristics of leukemic stem cells in acute leukemia and potential targeted therapies for their specific eradication

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Abstract

In acute myeloid leukemia (AML), a small cell population that contains stem cell features such as lack of differentiation, self-renewal potential, and drug resistance, can be identified. These so-called leukemic stem cells (LSCs) are thought to be responsible for relapse initiation after initial treatment leading to successful eradication of the bulk AML cell population. Since many studies have aimed to characterize and eliminate LSCs to prevent relapse and increase survival rates of patients, LSCs are one of the best characterized cancer stem cells. The specific elimination of LSCs, while sparing the healthy normal hematopoietic stem cells (HSCs), is one of the major challenges in the treatment of leukemia. This review focuses on several surface markers and intracellular transcription factors that can distinguish AML LSCs from HSCs and, therefore, specifically eliminate these stem cell-like leukemic cells. Moreover, previous and ongoing clinical trials of acute leukemia patients treated with therapies targeting these markers are discussed. In contrast to knowledge on LSCs in AML, insight into LSCs in acute lymphoid leukemia (ALL) is limited. This review therefore also addresses the latest insight into LSCs in ALL.

Keywords: Acute myeloid leukemia, acute lymphoid leukemia, leukemic stem cells, targeted therapy

INTRODUCTION

Acute leukemia is a rapidly progressing hematological malignancy that causes more than ten thousand deaths per year in the United States alone[1]. Acute leukemia can be subdivided into two main classes: acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL). AML is a heterogeneous malignancy characterized by the proliferation and accumulation of myeloid progenitor cells in the bone marrow (BM) and peripheral blood[1,2], while in ALL lymphoid progenitor cells accumulate in the BM and peripheral blood[3]. Based on the immunophenotype, ALL can be subdivided into different types; B-cell acute lymphoid leukemia (B-ALL) and T-cell acute lymphoid leukemia (T-ALL), with B-ALL being the most common type[4]. The median age of onset differs between AML and ALL. While ALL is most commonly diagnosed in children, AML occurs most frequently in patients older than 65 years of age[1,5]. Despite standard treatment with intensive cytotoxic induction chemotherapy and various clinical trials, five-year overall survival (OS) rates remain poor, especially in adults[6-8]. For treated AML patients between 60 and 70 years old, the OS rate is about 25%, and for ALL patients aged 70 years and older, it is only 15%. This is much lower compared to OS rates in children: around 80% in ALL and 70% in AML, depending on the risk group classification[9,10]. A major cause of dismal outcomes for both AML and ALL patients is the high relapse rate[7,8]. The prevention of relapse remains one of the most complicated challenges in the treatment of acute leukemia. However, the discovery of a rare so-called leukemia stem cell (LSC) population in AML has led to more insight into the mechanisms of relapse development and thereby novel therapeutic opportunities.

In the 1990s, a detailed investigation of AML subpopulations provided the first proof of a rare LSC population, with a CD34+/CD38- phenotype, within AML capable of establishing leukemia in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice: leukemic stem cells (LSCs)[11,12]. This subpopulation of cells shares several properties with normal hematopoietic cells (HSCs). For example, AML LSCs are, similar to HSCs, self-renewing cells that remain undifferentiated themselves but are capable of giving rise to both a stem cell copy and more differentiated progeny cells through mitotic cell division[13]. In addition, direct evidence shows that AML consists of three distinct LSC classes with heterogeneity in their self-renewal potential: short-term, long-term, and quiescent long-term LSCs[14]. Since HSC compartments have a similar hierarchical structure of heterogeneous cell classes, it is indicated that LSCs in AML originate from normal HSCs[12]. In concordance with these functional characteristics, it is suggested that AML LSCs share specific stem cell transcriptional programs with HSCs[15]. Altogether, this provides strong evidence for a hierarchical organization in AML with LSCs at the apex. Only these LSCs have the ability to initiate and fuel the disease, distinguishing them from more differentiated non-tumorigenic leukemic cells and healthy cells[13,16]. The stem cell features that distinguish LSC from healthy cells or more differentiated leukemic cells not only provide the capacity to initiate and maintain leukemia but are also thought to contribute to relapse. For example, drug-resistant properties due to changes in the expression of drug resistance genes are attributed to the stem cell phenotype[13,17-19]. The clinical relevance of LSCs is underlined by studies that showed an increased chance of relapse and worse overall survival in AML patients with a high CD34+/CD38- LSC frequency at diagnosis and after induction therapy compared to patients with a low LSC frequency[20]. These data suggest that eliminating LSCs during or after induction therapy will be crucial in improving the clinical outcome of AML patients.

Besides functional similarities, LSCs and HSCs also differ in many characteristics, such as cell surface protein expression or activation of intracellular signaling pathways, which may be exploited to specifically eliminate LSCs while sparing HSCs. Some of the key signaling pathways that play a role in the regulation of self-renewal, survival, proliferation, and differentiation are dysregulated in LSCs *vs.* HSCs. Examples of such signaling pathways include JAK/STAT, Wnt/β-catenin, Hedgehog, and Notch[21-24]. The dysregulation of these key signaling pathways in LSCs not only contributes to their oncogenic potential and cancer

progression, but some of them also contribute to drug resistance, illustrating the importance of therapeutically targeting these pathways.

Other factors suggested to be involved specifically in drug resistance of LSC and therefore promising targets are intracellular enzymes such as aldehyde dehydrogenase (ALDH) and histone deacetylase (HDAC)[25,26]. In addition, the overexpression of drug efflux transporters such as ATP-binding cassette transporters is also suggested to be an important intrinsic resistance mechanism (comprehensively reviewed in[27]), but treatment with specific inhibitors remains controversial for acute leukemia. Recently, there has been a revival of the research into the metabolic rewiring of resistant cancer cells. For LSCs specifically, the pathway of mitochondrial oxidative phosphorylation seems to be very distinct, as comprehensively reviewed by de Beauchamp *et al.*[28]. Extrinsic factors, including proteins involved in the cell-to-cell interactions between LSCs and the tumor microenvironment, could sensitize LSCs for eradication[29,30]. In addition to these intrinsic and extrinsic mechanisms, identification of aberrantly expressed surface markers on LSCs in AML is also a technique of interest in order to specifically eradicate these cells. Due to surface markers uniquely expressed on AML LSCs and not on HSCs, specific drug delivery to the rare population of LSCs is feasible without harming normal stem cells[31,32].

While knowledge on LSCs in AML has rapidly increased over the past few years, less is known about the nature of LSCs in ALL. There are even contradictory results on the existence of an LSC population in ALL. Several studies support a similar hierarchical organization in ALL as AML, while other studies provide evidence suggesting that the stochastic model may fit better[33,34]. This model states that all tumor cells are biologically equal and have the same tumor initiating potential. Their intrinsic characteristics cannot predict their behavior, so enrichment of these cells by sorting them based on these characteristics is impossible according to this model[16]. Therefore, besides summarizing the most important AML LSC specific markers and recent clinical trials targeting these markers, evidence and important markers of a stem cell population in ALL are also discussed.

LEUKEMIC STEM CELL TARGETS IN ACUTE MYELOID LEUKEMIA

AML LSCs cell surface protein targets

Similar to what is seen for AML LSCs, HSCs also have a CD34+/CD38- phenotype. The challenge in eliminating LSCs while sparing HSCs is therefore to find unique markers on LSCs that distinguish them from HSCs. Several studies have shown markers that are aberrantly expressed on LSC but not or very lowly expressed on normal HSC[32,35]. These markers can be used to isolate LSCs for further characterization, as well as monitored during therapy[20]. In addition, these markers are investigated for specific targeting by therapeutics, such as antibody-drug conjugates (ADCs) and chimeric antigen receptor (CAR)-T cells directed to these LSC-specific markers. The most important LSC specific cell surface markers are detailed below.

CD123

One of the surface markers that has been shown to be overexpressed in primary AML blasts and LSCs is CD123 [interleukin-3 receptor alpha (IL-3Rα)]. CD123 expression was not detectable on normal CD34+/CD38- hematopoietic cells, HSCs, discriminating LSCs from HSCs[36]. Despite the lack of CD123 expression on HSCs in healthy subjects, several other studies did find CD123 expression on HSCs and more differentiated hematopoietic cells such as monocytes[37,38]. For instance, NOD/SCID mice injected with patient cells from cord blood and BM revealed that not only human AML LSCs expressed CD123, but also a small proportion of normal human HSCs were positive for CD123[37]. However, since the percentage of CD123+ HSCs in the bone marrow was relatively low and only a relatively small part of HSCs expressed

CD123, most HSCs should not be harmed by CD123-targeted therapy[37].

Elevated levels of CD123 in AML are correlated to an increased number of leukemic blasts at diagnosis, a decreased chance to achieve complete remission and poor survival rates[39]. Moreover, high CD123 expression was associated with more cell cycle activity in the leukemic blasts, apoptotic resistance, and elevated signal transducer and activator of transcription 5 (STAT5) activation by IL-3[39]. The biological basis explaining this poor survival, when blasts have high CD123 expression, is enhanced signaling via the IL-3R in the CD123 overexpressing AML LSCs, resulting in increased proliferation and cell viability and decreased CXCR4 expression[30]. CXCR4 is a receptor expressed by HSCs/LSCs that interacts with stromal-derived factor 1 (SDF1), a chemokine constitutively expressed by BM stromal cells[40]. This interaction plays a major role in the homing and preserving of the stem cells in the BM niche. Downregulation of CXCR4 *in vitro* impaired the migration of LSCs to SDF1, suggesting that high CD123 expression and downregulated CXCR4 in LSCs releases them from the chemoprotective BM niche into the circulation[30]. A promising strategy to eliminate CD123-expressing AML LSCs could therefore be to combine CXCR4 antagonists with CD123 antibodies, since the antagonists would release more cells from the BM, leading to more effective targeting of LSCs by CD123 antibodies.

CD33

Another surface marker identified on LSCs is CD33, also known as sialic acid-binding Ig-like lectin 3[37]. Since CD33 shows similar homogeneous expression in relapsed AML samples as CD123, it was suggested that both these surface markers are promising drug targets[38]. Gemtuzumab ozogamicin (GO), a humanized anti-CD33 monoclonal antibody attached to a cytotoxic agent, has been used in the clinic and has shown clinical efficacy in the treatment of AML. However, since CD33, similar to CD123, shows expression on HSCs, healthy BM myeloid progenitor cells, and more differentiated myeloid cells, the risk of unwanted on-target off-tumor toxicity increases after targeting these molecules[38]. This is especially seen in ADCs targeting CD33, since the expression of CD123 is more restricted in healthy BM cells compared to CD33 expression[41]. In addition, the clinically effective GO revealed severe toxicities such as liver and hematological toxicities[41,42]. Therefore, targeting of more specific surface markers is required to reduce such toxicities.

T cell immunoglobulin mucin-3

T cell immunoglobulin mucin-3 (TIM-3), a transmembrane protein initially found on differentiated CD4+ Th1 and CD8+ Tc1 cells, is a membrane marker expressed on AML cells[43]. TIM-3 is expressed on multiple immune cells, such as regulatory T cells, natural killer cells, dendritic cells, monocytes, and macrophages[44-46]. Transcriptional profiling of LSCs and HSCs derived from human AML samples showed that TIM-3 is highly expressed on most LSCs, with the exception of acute promyelocytic leukemia LSCs, but not expressed on normal HSCs[47]. In addition, only TIM-3-positive AML cells, and not TIM-3 negative ones, were able to regenerate AML in immune-deficient mice. A similar differential expression of TIM-3 between LSCs and HSCs was observed in a study performing flow cytometry on primary human AML samples[48]. This differential expression allows for prospective separation of LSCs from normal HSCs, and it is also promising for the successful elimination of LSCs in AML[48]. Moreover, the number of TIM-3 expressing LSCs after allogeneic stem cell transplantation seems to be predictive for relapse[49].

That LSCs can effectively be eliminated by targeting TIM-3 is supported by *in vivo* experiments in human AML xenograft mice using the anti-human TIM-3 mouse antibody named ATIK2a. This antibody induces antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, which resulted in the effective eradication of LSCs without harming HSCs. The outcome of this was a strong decrease in

leukemic burden in treated mice[47]. Altogether, these studies provide evidence that TIM-3 is a promising target in the elimination of LSCs and suggest that targeting of TIM-3 results in fewer side effects compared to the targeting of, e.g., CD33.

C-type lectin-like molecule-1

C-type lectin-like molecule-1 (CLL-1), a transmembrane glycoprotein, was first identified on AML cells in 2004[50]. CLL-1 is expressed in 92% of primary AML samples[50] and expressed on LSCs in the majority of AML patients, but it is absent on HSCs from healthy and regenerating BM from patients who received chemotherapeutic treatment[51,52]. It was recently published that CLL-1 expression can also be bimodal in AML samples[53], which warrants further investigation into effective elimination of LSCs including those negative for CLL-1. Despite this, CLL-1 is still a promising target in the treatment of AML. Zheng *et al.*[54] studied the efficacy and safety of an anti-CLL-1-ADC in which a CLL-1 antibody is conjugated via a self-immolative disulfide linker to a pyrrolobenzodiazepine (PBD) dimer. Xenograft mice and cynomolgus monkeys were treated with this anti-CLL-1 ADC and showed an effective decrease in AML cells. Despite the fact that CLL-1 is expressed on healthy myeloid progenitors, its expression pattern is more restricted on healthy cells than that of CD33, resulting in less toxicity and faster recovery from side effects such as cytopenia[54]. Anti-leukemic effects were also observed in xenografted mice engrafted with human AML and treated with CAR-T cells targeting CLL-1[52]. The results show both *in vitro* and *in vivo* compelling anti-leukemic effects.

CD47

CD47 is a surface marker widely expressed on both hematopoietic cells and other cell types[55]. The interaction between CD47 and SIRPα, a protein expressed on phagocytic cells such as dendritic cells and macrophages, leads to inhibition of phagocytosis[56]. The CD47 surface marker is, compared to the other mentioned surface markers, not as stem cell-specific and therefore not used to identify and monitor stem cells. However, since it was found that CD47 has elevated expression on AML LSCs compared to normal HSCs, blocking monoclonal antibodies have been used in multiple studies as a strategy to eliminate LSCs[56,57]. The results show that these blocking anti-CD47 antibodies enable phagocytosis, resulting in the eradication of LSCs without affecting normal cells[55-57].

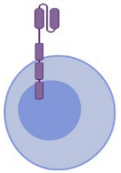
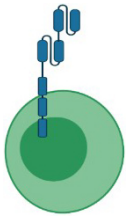
Targeting LSCs via one protein is a big challenge, since even a low expression of a cell surface marker on HSCs leads to unwanted toxicities; thus, more specific targeting of LSCs is required to prevent this. One option to increase efficacy and decrease toxicity is by targeting surface marker combinations that are highly co-expressed on AML cells but not co-expressed on healthy cells. Haubner *et al.*[38] showed that this is valid for the CD33/TIM-3 and CLL-1/TIM-3 combinations.

Different therapies have been developed to target these surface markers specifically expressed on LSCs [Table 1]. Besides the described surface markers, other commonly investigated surface markers are also included in the table.

The surface markers in Table 1 are all surface markers of AML LSCs that have been investigated by many different studies. Recently, two novel and less thoroughly investigated candidate AML LSC surface markers have been identified. First, CD9, a member of the tetraspanin family, was found to be highly expressed on LSCs in AML patients, and CD9-positive AML cells were capable of initiating AML *in vivo*[87,88]. Since CD9 is hardly expressed on normal HSCs, it was suggested that this surface marker could be a promising new target for the eradication of LSCs. Second, AML cells positive for c-MPL, a thrombopoietin receptor regulating processes such as self-renewal and HSC proliferation, showed more chemoresistance than the c-

Table 1. Examples of recently developed therapies against AML LSC surface markers

Therapy type	Surface marker	Drug name	Ref.
Monoclonal antibodies			
Naked antibody 	CD123	CSL362 KHK2823	Busfield *et al.*[58] 2014 Akiyama *et al.*[59] 2015
	CD33	Lintuzumab	Sutherland *et al.*[60] 2009
	TIM-3	ATIK2a MBG453	Kikushige *et al.*[47] 2010 Schürch[61] 2018
	CD96	MSH-TH111e	Gramatzki *et al.*[62] 2016
	CD99	H036-1.1	Chung *et al.*[63] 2017
	CD44	H90	Gadhoum *et al.*[64] 2016
	CD47	B6H12.2 BRIC126 Hu5F9-G4 CC-90002	Majeti *et al.*[56] 2009 Liu *et al.*[57] 2015 Narla *et al.*[55] 2017
Antibody-drug conjugate 	CD123	IMGN632 SGN-CD123A	Kovtun *et al.*[65] 2018 Li *et al.*[66] 2018
	CD33	Gemtuzumab ozogamicin (MyloTarg®) SGN-CD33A	Gottardi *et al.*[67] 2020 Kung Sutherland *et al.*[68] 2013
	CLL-1	Anti-CLL-1-ds-PBD	Zheng *et al.*[54] 2019
	CD25	ADCT-301	Flynn *et al.*[69,70] 2014, 2016
Radioimmunoconjugate	CD123	111In-DTPA-NLS-CSL360	Gao *et al.*[71] 2016
	CD33	225Ac-lintuzumab	Jurcic *et al.*[72] 2013
Bispecific antibodies			
Dual-affinity re-targeting antibody (DART) 	CD123xCD3	Flotetuzumab	Chichili *et al.*[73] 2015
Bi-specific T-cell Engager (BiTE)	CD123xCD3	BiTE(CSL263/OKT3)	Hutmacher *et al.*[74] 2019

	CD33xCD3	AMG-330 AMG-673	Krupka *et al.*[75] 2014 Subklewe *et al.*[76] 2019
T cell-dependent bispecific (TDB) antibody	CLL-1xCD3	CD3/CLL-1 TDB MCLA-117	Leong *et al.*[77] 2017 van Loo *et al.*[78] 2019
			
Other bispecific antibodies	CD47xCD33	HMBD004	Boyd-Kirkup *et al.*[79] 2017
Chimeric antigen receptor (CAR)-T cells			
	CD123	MB-102	Mardiros *et al.*[80] 2013
	CD33	CART-33	Kenderian *et al.*[81] 2015
	CLL-1	CLL-1 CAR-T cells	Wang *et al.*[52] 2018
	CD44v6	CD44v6.CAR28z	Casucci *et al.*[82] 2013
	CD7	CD7 CAR-T cells	Gomes-Silva *et al.*[83] 2019
Other			
TRAIL	CLL-1	CLL-1:TRAIL	Wiersma *et al.*[84] 2015
	CD25	IL2-TRAIL	Madhumathi *et al.*[85] 2017
SAR-transduced T cells	CD123	Anti-E3-anti-CD123 taFv	Benmebarek *et al.*[86] 2021
	CD33	Anti-E3-anti-CD33 taFv	Benmebarek *et al.*[86] 2021

Figures created with BioRender.com. SAR: Synthetic agonistic receptor; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand; CD44v6: CD44 variant domain 6; taFv: tandem single chain variable fragment.

MPL negative AML cell population in a mouse leukemic model[89]. Moreover, the c-MPL+ cells had a higher self-renewal potential and were significantly better at initiating AML *in vivo* compared to the c-MPL- cell population. Although these results suggest that c-MPL could be a potential target to eradicate AML LSCs, more research is needed regarding unwanted toxicities since c-MPL is also a long-term HSCs marker[90].

Signal transduction pathways and transcription factors involved in AML LSC survival

Besides the previously described surface markers, there are intracellular proteins such as transcription factors that are differentially expressed between LSCs and HSCs[91]. Several of these factors are involved in drug resistance mechanisms, making them important therapeutic targets for elimination of AML LSCs.

JAK/STAT signaling pathway

High CD123 expression is associated with elevated STAT5 activation by IL-3, suggesting that the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway plays a role in the fate of LSCs [Figure 1A][39]. The JAK family of intracellular non-receptor tyrosine kinases can be activated via extracellular cytokine or growth factor binding, resulting in the phosphorylation and activation of STAT proteins[92]. This STAT protein family consists of transcription factors that interfere with proliferation, differentiation, and apoptosis. Previous research has shown that STAT3 and STAT5 are constitutively activated in AML leukemic blasts, which is not seen in HSCs[93]. This may contribute to the uncontrolled proliferation of these blasts and resistance to chemotherapy-induced apoptosis. The role of JAK/STAT signaling in AML LSCs growth and survival was investigated several years later by evaluating the expression levels of JAK and STAT in AML patient samples at diagnosis and relapse[94]. An ATP competitive inhibitor of JAK1/2 kinases named AZD1480 was used in both *in vitro* and *in vivo* experiments to analyze its effect on AML stem/progenitor cells. Inhibitor treatment of AML CD34+ cells *in vitro* showed decreased levels of JAK2 and STAT3/5 activity and reduced AML CD34+ cell proliferation and survival, but it did not affect normal CD34+ cells[94]. Similar results were seen in NOD/SCID mice treated with AZD1480: the number of AML LSCs was reduced, but normal human HSC numbers were not affected. To further investigate the role of JAK1, JAK2, STAT3, and STAT5 in CD34+ AML cells, an RNA interference-mediated knockdown of these proteins was performed. JAK2, STAT3, and STAT5 knockdown resulted in a significant decrease of colony-forming cells, cell numbers, and survival, while this was not observed with downregulation of JAK1, indicating that inhibition of JAK2 is more effective in decreasing growth and survival of AML CD34+ cells than inhibition of JAK1.

Nuclear factor-kappa B signaling pathway

Nuclear factor-kappa B (NF-κB) is a proinflammatory transcription factor that plays an essential role in cellular processes such as proliferation, survival, stress responses, and inflammation[95]. NF-κB is suggested to be involved in drug resistance, as NF-κB has anti-apoptotic activity and increased levels of NF-κB have been seen after chemotherapy and radiotherapy[96,97]. Electrophoretic mobility shift assays on primary AML samples showed constitutively activated NF-κB in AML LSCs. In contrast, in normal human stem/progenitor cells, there was no NF-κB expression[23]. Multiple studies have investigated the effect of NF-κB inhibitors on AML (stem) cells. For example, AML LSCs treated with MG-123, an NF-κB inhibitor, initiated cell death *in vitro*[23]. Micheliolide (MCL), a natural sesquiterpene lactone, had cytotoxic effects on LSCs via the inhibition of NF-κB[98]. *In vitro* MCL treatment initiated apoptosis in AML LSCs but not in normal HSCs. *In vivo*, treatment with DMAMCL, the pro-drug form of MCL, improved survival rates of NOD/SCID mice engrafted with human AML[98]. A third NF-κB inhibitor, BMS-345541, conferred an altered expression of genes involved in a previously described 17-gene LSC score to primary AML patient samples[15,95]. This gene signature contains 17 stemness-related genes differentially expressed between LSC-positive and LSC-negative AML samples. The NF-κB pathway is illustrated in Figure 1B, including drugs recently used in clinical trials targeting this pathway. In addition, it has to be noted that this pathway may

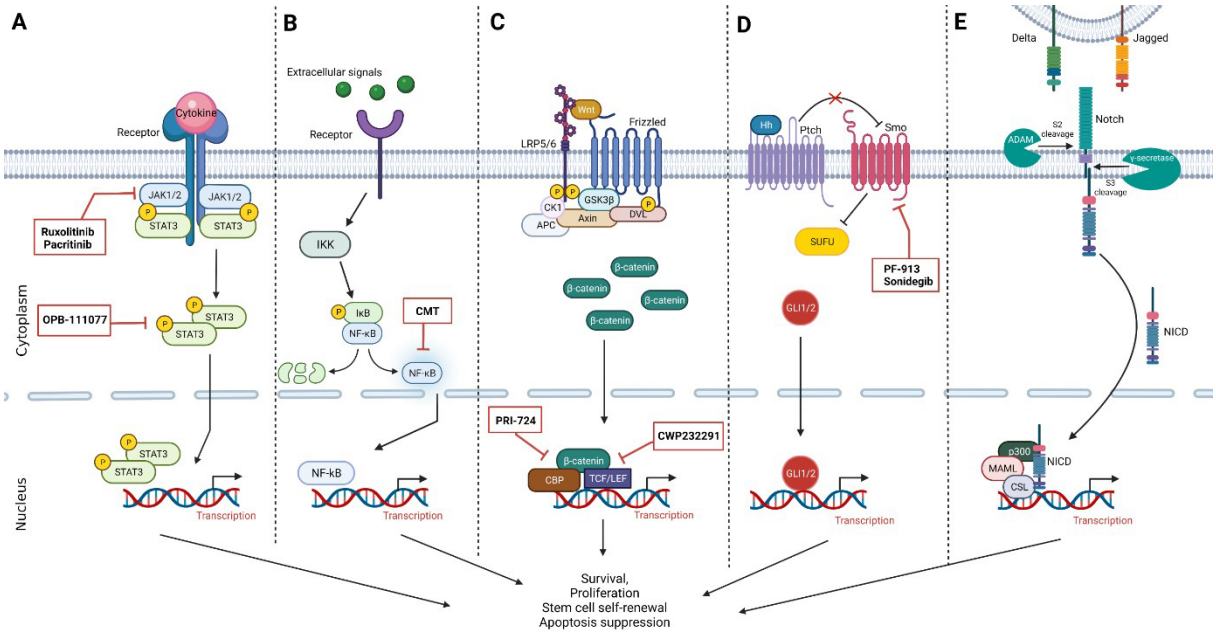


Figure 1. Intracellular signaling pathways dysregulated in AML LSCs, including agents recently used in clinical trials inhibiting pathway activity. (A) JAK/STAT signaling pathway. Therapeutic agents either target elevated JAK1/2 levels or constitutively active STAT3. (B) NF-κB signaling pathway. Therapeutic agents recently used in clinical trials directly inhibit the constitutively activated NF-κB. (C) Wnt/β-catenin signaling pathway. Therapeutic targets prevent constitutive activation by inhibiting the interaction between β-catenin and CBP or TCF. (D) Hh signaling pathway. Small molecule inhibitors target Smoothened (Smo), decreasing pathway activation. (E) Notch signaling pathway. There are currently no clinical trials inhibiting or activating the Notch pathway as a treatment in AML patients. Created with BioRender.com. AML: Acute myeloid leukemia; LSCs: leukemic stem cells; JAK/STAT: Janus kinase/signal transducer and activator of transcription; NF-κB: nuclear factor-kappa B; CBP: CREB-binding protein; TCF: T-cell factor; Hh: Hedgehog.

also be linked to the PI3K/Akt/mTOR pathway, which has also been suggested to be involved in the drug resistance of AML LSC[99].

Wnt/β-catenin signaling pathway

The Wnt/β-catenin signaling pathway is a highly conserved signaling pathway involved in the development and tissue homeostasis[100]. The Wnt/β-catenin pathway plays a role in the cellular processes of HSCs, including cell proliferation, differentiation, survival, and stem cell renewal[101]. The transcriptional coactivator β-catenin plays a central role in this pathway; when the Wnt ligand binds to its cognate receptor Frizzled, β-catenin degradation is blocked, causing an accumulation of β-catenin and subsequent translation to the nucleus. There, β-catenin binds to nuclear transcription factors that belong to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, which recruits coactivators including the CREB-binding protein (CBP), resulting in the transcription of target genes involved in self-renewal and proliferation[102]. A study evaluating the activity of Wnt/β-catenin signaling by transfecting AML and normal progenitors with a TCF/LEF reporter construct, found that in the majority of the AML samples, the TCF/LEF pathway was constitutively active, which was supported by other studies showing overexpression of β-catenin both in AML cell lines and patient samples[101,103]. Moreover, the promoter regions of Wnt pathway inhibitor genes were frequently methylated in cell lines, and in 89% of AML patient samples with normal cytogenetics one or more of these inhibitor genes were methylated. Methylation of two Wnt pathway inhibitor genes named *sFRP2* and *sFRP5* was associated with elevated relapse risk, suggesting that enhanced Wnt activity has adverse outcomes in AML patients with normal karyotypes[104].

Wang *et al.*[105], using an AML mouse model, showed that LSCs require β-catenin in order to maintain their self-renewal capacity. Several studies have shown the overexpression of β-catenin in LSCs; however, the expression of β-catenin in normal HSCs was also observed[106]. Cobas *et al.*[107] provided evidence that β-catenin did not show to be crucial for self-renewal of adult HSCs, since depletion of β-catenin in mice showed no impairment in hematopoiesis and lymphopoiesis. Targeting β-catenin is therefore suggested to be promising in the eradication of LSCs, while sparing HSCs [Figure 1C]. Nevertheless, targeting the Wnt/β-catenin pathway remains a challenge due to its complexity. First, mammals contain 19 different Wnt ligands and over 15 Frizzled receptors and co-receptors. In addition, targeting β-catenin is also complex since it can bind to many other transcription factors besides TCF/LEF[108]. Numerous small molecule inhibitors or antagonists with different targets within the Wnt/β-catenin pathway have been investigated, including inhibitors targeting the interaction between β-catenin and TCF[109]. Treatment with these inhibitors and antagonists is indicated to be relevant in LSC depletion by impairing their self-renewal capacity.

Hedgehog and Notch signaling pathways

Besides the Wnt/β-catenin pathway, the Hedgehog (Hh) and Notch signaling pathways are also highly evolutionarily conserved pathways involved in the development and tissue homeostasis[110]. A study in zebrafish treated with a Hh inhibitor suggested that Hh signaling is necessary for HSC homeostasis and differentiation[111]. A few years later, a study addressed Hh function specifically in adult HSCs, revealing that the deletion or overexpression of Smoothened, a G-protein coupled receptor playing a key role in Hh signaling, did not affect adult HSC self-renewal *in vivo*[112]. Studies investigating Notch signaling in HSCs showed controversial results as well. Notch signaling is activated in HSCs, but it decreases when HSCs differentiate. Inhibition of Notch signaling demonstrated increased HSC differentiation *in vitro* and depleted HSC levels *in vivo*, indicating that Notch signaling is essential for the self-renewal of HSCs[113]. This is contradictory to the results of follow-up studies that showed that inhibition of Notch signaling had no effect on HSCs[114,115].

Aberrant Notch and Hh signaling has been detected in AML LSCs. LSCs that exhibited active Hh signaling showed enhanced survival and chemoresistance[116]. This, together with the knowledge that inhibiting Hh does not affect HSCs[112], suggests that targeting Hh in AML patients could specifically eliminate AML LSCs [Figure 1D]. This idea was supported by a study that showed induction of apoptosis in CD34+ leukemic cells after treatment with a Hh neutralizing antibody or Smoothened antagonist[116]. Besides Hh signaling, the role of Notch signaling has also been investigated in AML, showing controversial results. Depending on the context, Notch signaling could exhibit both oncogenic and tumor suppressor functions *in vivo*[117]. In AML, Notch signaling has been shown to be mainly tumor suppressive. For instance, CD34+/CD38- LSCs, harvested from an MLL-AF9-driven AML mouse model, contained silenced Notch activity[118]. Both *in vivo* and *in vitro*, activation of the Notch pathway, by a gain of function models or treatment with a Notch ligand, respectively, led to decreased proliferation and increased apoptosis of this CD34+/CD38- LSC population[118]. However, despite several studies showing a tumor suppressive role of Notch signaling in AML LSCs, there were also studies providing evidence for an oncogenic role. For example, an oncogenic role of Notch signaling was observed in a pre-leukemic acute promyelocytic leukemia mice model[119]. In addition, crosstalk between the Wnt/β-catenin and Notch pathways displayed a promoting role of Notch signaling in AML development[120]. When β-catenin is activated, osteoblasts in the BM start to express the Notch ligand Jagged-1, resulting in activated Notch signaling in pre-leukemic hematopoietic stem/progenitor cells[120]. This led to the malignant transformation of these cells, providing evidence that activated Notch has an oncogenic function. However, in contrast to these results, constitutive low Notch and high Wnt signaling in LSCs was demonstrated to play a role in maintaining AML[121]. This result

suggests that promoting Notch signaling while blocking Wnt signaling could be a promising approach to eliminate LSCs in AML. The Notch signaling pathway and a promising therapeutic agent recently used in clinical trials is shown in Figure 1E.

In addition to the described signaling pathways, there are many other factors identified as being promising LSC targets, including miRNAs[122]. Examples of the most important markers and therapeutics targeting them are shown in Table 2.

LEUKEMIC STEM CELLS IN ACUTE LYMPHOID LEUKEMIA

Although AML is proven to be maintained by a rare population of LSCs, for ALL it is not clear if it is organized similarly. Several studies support a similar hierarchical organization in ALL as AML, while other studies provide evidence that contradicts this.

Leukemic stem cells in B-ALL

Two early studies from Cox *et al.*[149,150] evaluated the long-term proliferation of childhood B-ALL cells *in vitro* and *in vivo*. The expression of several surface markers, such as CD34, CD38, CD19, CD133, and CD10, on the B-ALL cells were investigated on their potential to initiate B-ALL. Only a minority of B-ALL cells, those with a primitive CD34+/CD10-/CD19–/CD38- phenotype, were capable of engrafting B-ALL in NOD/SCID mice. Since CD19 is known to be a B-lymphocyte antigen and CD10 a marker of lymphocytic differentiation, this result suggests that B-ALL arises, as in AML, from a primitive immature cell instead of a committed B cell[34,149]. A few years later, this group performed a follow-up study on the expression of the primitive cell antigen CD133. They found that only cells within the CD133+/CD19- and CD133+/CD38- phenotypes were capable of initiating B-ALL in children, which supported their previous findings[150,151].

Besides studies indicating that only cells with a primitive phenotype are able to engraft B-ALL in NOD/SCID mice, several other studies found contradictory results showing that exclusively the more mature CD19+ B-ALL cells were capable of engrafting[152,153]. In this latter study, both the CD34+/CD38-/CD19+ and the CD34+/CD38+/CD19+ B-ALL cell populations had the capacity to engraft B-ALL. Furthermore, in high-risk childhood ALL patients, including patients with *MLL* gene rearrangement, blasts cells were within three different maturation stages (CD34+CD19-, CD34+CD19+ and CD34-CD19+), which all had the capacity to re-establish and reconstitute the original leukemia phenotype in NOD/SCID mice[154]. That cells within different maturation stages have the capacity to engraft B-ALL and contain stem cell activity was confirmed by showing that both CD19+/CD20- and CD19+/CD20+ cells are capable of B-ALL engraftment. However, a study specifically looking at *MLL-AF4*+ infant B-ALL showed that exclusively the more mature CD34+CD19+ and CD34-CD19+ B-ALL cell populations could engraft[155]. Similar results were seen in standard-risk patients, such as patients with a *TEL/AML1* fusion gene, in whom engraftment of B-ALL was restricted to cells containing the CD19+ phenotypes[154]. These controversial findings highlight that the LSCs in B-ALL are heterogeneous and indicate that several cytogenetic aberrations are involved in driving LSCs.

Since in AML the enrichment of LSCs is well established and the CD34+CD38- fraction is suggested to contain the most important LSCs[17], the surface markers CD34 and CD38 have also often been used to enrich for LSCs in B-ALL[156]. However, there is a highly dynamic expression of CD34 and CD38 on leukemia-initiating cells in B-ALL[157], which could be an explanation for the controversial results found in the above-mentioned studies and also suggests a non-hierarchical organization of B-ALL.

Table 2. Examples of recently developed drugs targeting differentially expressed intracellular pathways and other factors in AML LSCs

Marker	Target	Drug type	Drug name	Ref.
Signaling pathways				
JAK/STAT	JAK1/2	ATP competitive inhibitor	AZD1480	Cook *et al.*[94] 2014
	JAK1/2	ATP competitive inhibitor	Ruxolitinib	Cook *et al.*[94] 2014
	JAK2	Small molecule inhibitor	Pacritinib	Balaian *et al.*[123] 2016
	STAT3	Antisense oligonucleotide	AZD9150	Shastri *et al.*[124] 2018
	STAT3	Small molecule inhibitor	OPB-111077	Wilde *et al.*[125] 2019
	STAT3	dODN competitive inhibitor	CpG-STAT3dODN	Zhang *et al.*[126] 2016
	STAT5	SH2 domain inhibitor	AC-4-130	Wingelhofer *et al.*[127] 2018
Wnt/β-catenin	CBP/β-catenin	Small molecule inhibitor	PRI-724	Jiang *et al.*[128] 2018
	CBP/β-catenin	Small molecule inhibitor	CWP232228	Benoit *et al.*[129] 2017
	β-catenin/TCF	Small molecule inhibitor	CWP232291	Kim *et al.*[101] 2011
	β-catenin	Small molecule inhibitor	BC2059	Fiskus *et al.*[130] 2015
	CK1α	Agonist	Pyrvinium	Fong *et al.*[131] 2015
Notch	Notch2	Agonist	Dll4-Fc	Lobry *et al.*[118] 2013
	Notch1	Agonist	NMHC	Ye *et al.*[132] 2016
	Notch1/2	Agonist	AZA	Dongdong *et al.*[133] 2019
	γ-secretase	GSI	BMS-906024	Arenas *et al.*[134] 2018; Grieselhuber *et al.*[119] 2013
Hedgehog	Smoothened	Small molecule inhibitor	PF-913	Fukushima *et al.*[135] 2016
	Smoothened	Small molecule inhibitor	Sonidegib	Li *et al.*[136] 2016
	Smoothened	Small molecule inhibitor	Saridegib	Lim *et al.*[137] 2015
	GLI1/2	Small molecule inhibitor	GANT-61	Long *et al.*[138] 2016
NF-κB	IKK	Small molecule inhibitor	BMS-345541	Reikvam[95] 2020
	NF-κB	GSL	Micheliolide	Ji *et al.*[98] 2016
	NF-κB	NSAID	CMT	Strair *et al.*[139] 2008
HDAC	HDAC	Benzamide-type inhibitor	Entinostat	Zhou *et al.*[140] 2013

	HDAC	Pan inhibitor	Panobinostat	Fiskus *et al.*[130] 2015
	HDAC	Pan inhibitor	Pracinostat	Novotny-Diermayr *et al.*[141] 2012
	HDAC	Benzamide-type inhibitor	Chidamide	Li *et al.*[142] 2015
Other				
ALDH	ALDH2	Non-specific inhibitor	Disulfiram	Yang *et al.*[143] 2020
	ALDH1/3	Competitive inhibitor	DIMATE	Venton *et al.*[144] 2016
CXCR4	CXCR4	Small molecule inhibitor	Plerixafor	Tavor *et al.*[145] 2008
	CXCR4	Small molecule inhibitor	BL-8040	Abraham *et al.*[146] 2017
	CXCR4	Small molecule inhibitor	AMD3465	Zeng *et al.*[147] 2009
	CXCR4	Monoclonal antibody	BMS-936564	Kuhne *et al.*[148] 2013

dODN: Decoy oligodeoxynucleotide; CBP: CREB-binding protein; Sam68: SRC-associated in mitosis 68; CK1α: casein kinase 1α; Dll4-Fc: delta-like 4 extracellular domain fused to the IgG-Fc-fragment; NMHC: *N*-methylhemeanthidine chloride; AZA: azelaic acid; GSI: gamma-secretase inhibitor; GLI: glioma-associated oncogene homolog; IKK: IκB kinase; GSL: guaianolide sesquiterpene lactone; CMT: choline magnesium trisalicylate; NSAID: non-steroidal anti-inflammatory drug; DIMATE: dimethyl ampal thiolester; HDAC: histone deacetylase; ALDH: aldehyde dehydrogenase.

Taken together, accumulating evidence shows that there are several different ALL LSCs with a variety of immunophenotypes, making it impossible to isolate these cells based on their surface markers. Moreover, this indicates that there is not just a rare subset of B-ALL cells with an enhanced leukemogenic potential, and that the stochastic model, rather than the hierarchical LSC model observed in AML, applies for B-ALL[33,158].

Leukemic stem cells in T-ALL

The identification of LSCs in human T-ALL is, as in B-ALL, a major challenge. Cox *et al.*[159] tried to identify LSCs in pediatric T-ALL patients by performing *in vitro* suspension culture assays and *in vivo* NOD/SCID mice model experiments. T-ALL cells with the expression of CD34, in combination with CD4 and CD7, were investigated for their potential to be LSCs. CD4 is a co-receptor of the T cell receptor and CD7 is a marker of early T cell differentiation[160,161]. Exclusively cells within the rare CD34+/CD4- and CD34+/CD7- subfractions were capable of T-ALL engraftment in mice, suggesting that pediatric T-ALL arises from cells with a primitive immunophenotype, and that T-ALL is similarly organized as in AML[159]. Contradicting results were found in a study that investigated cortical/mature T-ALL patient samples. In these T-ALL patients, the LSC activity was limited to cells within the CD34+/CD7+ subpopulation both *in vitro* and *in vivo*, while the primitive CD34+/CD7- cells only grew out into normal HSCs[161].

The previous two studies suggest that LSC activity in T-ALL is limited to the CD34+ phenotype. However, it was revealed that the CD34+ fraction in some T-ALL samples also contained LSC activity, while in other samples the LSC activity was in the CD34- population[162]. This indicates that, as seen in B-ALL, CD34

is not a universal marker to identify LSCs in all adult T-ALL patients. Interestingly, Chiu *et al.*[162] found that a CD7+/CD1a- T-ALL cell subset was enriched for LSC activity, suggesting that adult T-ALL arises from immature thymocytes and is organized as a hierarchical CSC model. Moreover, these CD7+/CD1a- cells were shown to be resistant to glucocorticoids such as dexamethasone and prednisone[162]. These drugs are commonly used for the treatment of ALL, and particularly in T-ALL, resistance to glucocorticoids is the most important driver of treatment failure[163]. Together, these results indicate that the CD7+/CD1a- T-ALL cell fraction is functionally different from the bulk of the T-ALL, and that it might be important to eliminate these cells to overcome treatment failure.

Besides studies investigating possible surface markers in T-ALL, there are also studies focusing on signaling pathways that could play a key role in T-ALL relapse. Since the majority of patients have T-ALL with oncogenic *Notch1* mutations, a recent study explored the significance of interleukin-7 receptor (IL-7R) signaling, a transcriptional target of Notch1, in LSC potential in T-ALL cell lines, human pediatric samples, and one adult T-ALL sample[164]. Expression of functional IL-7R is crucial for the emergence of Notch1-induced T-ALL, and IL-7R was demonstrated to be a biomarker for LSCs in T-ALL. Besides human T-ALL, IL-7R is also essential in B-ALL cells containing LSC activity and promoting B-ALL progression, suggesting that targeting IL-7R could prevent relapse in both pediatric T-ALL and B-ALL patients[164].

Despite a less well-characterized ALL organization compared to AML, there are several clinical trials that focus on targeting populations of cells that have a high initiating potential in ALL such as CD34+CD38+CD19+ cells[153]. Besides these ALL trials, numerous clinical trials targeting AML LSCs are currently active.

CLINICAL TRIALS TARGETING LSCS

Many different surface markers, transcription factors, and other factors have been described to be differentially expressed between HSCs and LSCs. These are all promising targets in the treatment of acute leukemia, and therefore currently, several clinical trials are investigating the effect of drugs targeting them. The clinical trials in Supplementary Table 1 are examples of recent clinical trials investigating the effects of these anti-LSC compounds, either as a monotherapy or in combination with other therapies.

Summary and important remarks of recent clinical trials targeting LSC-specific markers

Supplementary Table 1 shows many clinical trials focusing on the markers mentioned in the previous sections. Most of these trials, investigating novel therapeutic compounds targeting AML LSCs, are phase I or II trials; however, some compounds are already in phase III: CD123/CLL-1 CAR-T cells, Hh pathway inhibitor PF-913, and HDAC inhibitors pracinostat and panobinostat. Besides the majority of these clinical trials focusing on LSCs in AML, a few have investigated compounds targeting cells capable of initiating B-ALL or T-ALL: anti-CD25 ADC ADCT-301, bispecific anti-CD19/CD3 BiTE blinatumomab, anti-CD19 CAR-T cells, anti-CD7 CAR-T cells, JAK1/2 inhibitor ruxolitinib, Notch inhibitor BMS-906024, and CXCR4 antagonists. The use of CAR-T cells is an often-used strategy to target surface markers in ALL as well as AML. Interestingly, the use of CAR-T cells is one of the few therapeutic strategies in clinical trials with patients under the age of 18. Clinical trials using other compounds or targeting LSC-related pathways are exclusively performed in adults.

Although some clinical trials are investigating the safety and efficacy of novel therapeutics as a monotherapy, most of the compounds are tested in combination with other drugs, such as chemotherapy and hypomethylating agents. A few clinical trials have been terminated [Supplementary Table 1], either due to a lack of efficacy or slow enrollment, but not because toxicities were seen. Despite this, many recent

clinical trials are still recruiting and some clinical trials targeting LSC-related surface markers or pathways have even been completed with published results.

Published results from recent clinical trials targeting LSC-specific markers

Results from two recent clinical trials involving the targeting of AML LSC surface markers have been published. The clinical trial evaluating the safety and efficacy of anti-CD33 (ADC SGN-CD33A) in newly diagnosed AML patients (NCT02326584) demonstrated that ADC SGN-CD33A is safe both as a monotherapy and in combination with standard high-dose cytarabine (HiDAC) therapy. As a single agent, it resulted in on-target myelosuppression with mild non-hematologic side effects when administered after chemotherapy and/or after allogeneic stem cell transplantation[165]. Besides CD33, the surface marker CD47 has been tested as an LSC target. Relapsed/refractory AML patients treated with the anti-CD47 monoclonal antibody Hu5F9-G4 (NCT02678338) showed decreased hemoglobin levels in all patients and difficulties with blood compatibility testing[166]. This could be explained by the fact that CD47 is also expressed on red blood cells, suggesting that HU5F0-G4 is capable of clearing these red blood cells. This therapeutic can only be used safely by carefully monitoring patients receiving Hu5F9-G4.

To investigate the effect of targeting CD19+ B-ALL blasts, blinatumomab, a bispecific anti-CD19/CD3 BiTE, has been used in multiple clinical trials. Results from a phase III clinical trial (NCT02013167) have shown that, compared to chemotherapy, blinatumomab results in improved minimal residual disease remission and longer overall survival in adult B-ALL patients[167,168], suggestive for eradicating B-ALL LSCs. In addition, a recent study (NCT02143414) showed benefits for older patients with newly diagnosed Ph chromosome-negative B-ALL, including patients with poor-risk cytogenetics[169]. Besides antibody therapy, CAR-T therapy targeting CD19 has been shown to be effective in B-ALL[170,171], although it is not specifically shown to be particularly directed against LSCs. An overview of all recent therapeutic strategies targeting LSC-specific surface markers in both AML and ALL is shown in Figure 2.

In addition to clinical trials investigating drugs against the LSC surface markers, results from several clinical trials targeting LSC-related signaling pathways [Figure 1] have been published. First, two JAK inhibitors, ruxolitinib and pacritinib, showed promising results. Patients with relapsed/refractory AML treated with ruxolitinib (NCT00674479) revealed that this JAK inhibitor has limited toxicities and modest anti-leukemic activity[172]. Combining the JAK inhibitor pacritinib with chemotherapy in AML patients with *FLT3* mutations (NCT02323607) gave similar results as ruxolitinib, since it was well tolerated and showed anti-leukemic activity[173,174]. Second, Hh pathway inhibitors, such as PF-913 and sonidegib targeting Smoothened, have been investigated. In a phase II study (NCT01546038), the combination of PF-913 and low-dose cytarabine improved the overall survival of AML patients when compared to low-dose cytarabine alone. This improved outcome was predominantly seen in patients with secondary AML[175]. Notably, sonidegib combined with the hypomethylating agent azacitidine (NCT02129101) did not show such an improvement, compared to single azacitidine treatment, in patients with previously treated or advanced myeloid malignancies. However, in the relapsed/refractory AML patient population, the combination of sonidegib with azacitidine showed an increase in OS rates and an absence of progression[176]. Finally, the Wnt/β-catenin pathway inhibitor CWP232291 has been investigated as a single agent in relapsed/refractory AML patients (NCT01398462). This inhibitor was well tolerated but only minimally effective[173]. Despite this disappointing result, CWP232291 has great potential in the elimination of LSCs when used in combination with other drugs such as chemotherapy, since this Wnt/β-catenin pathway inhibitor may be very efficient in killing leukemia cells with self-renewal potential such as LSCs.

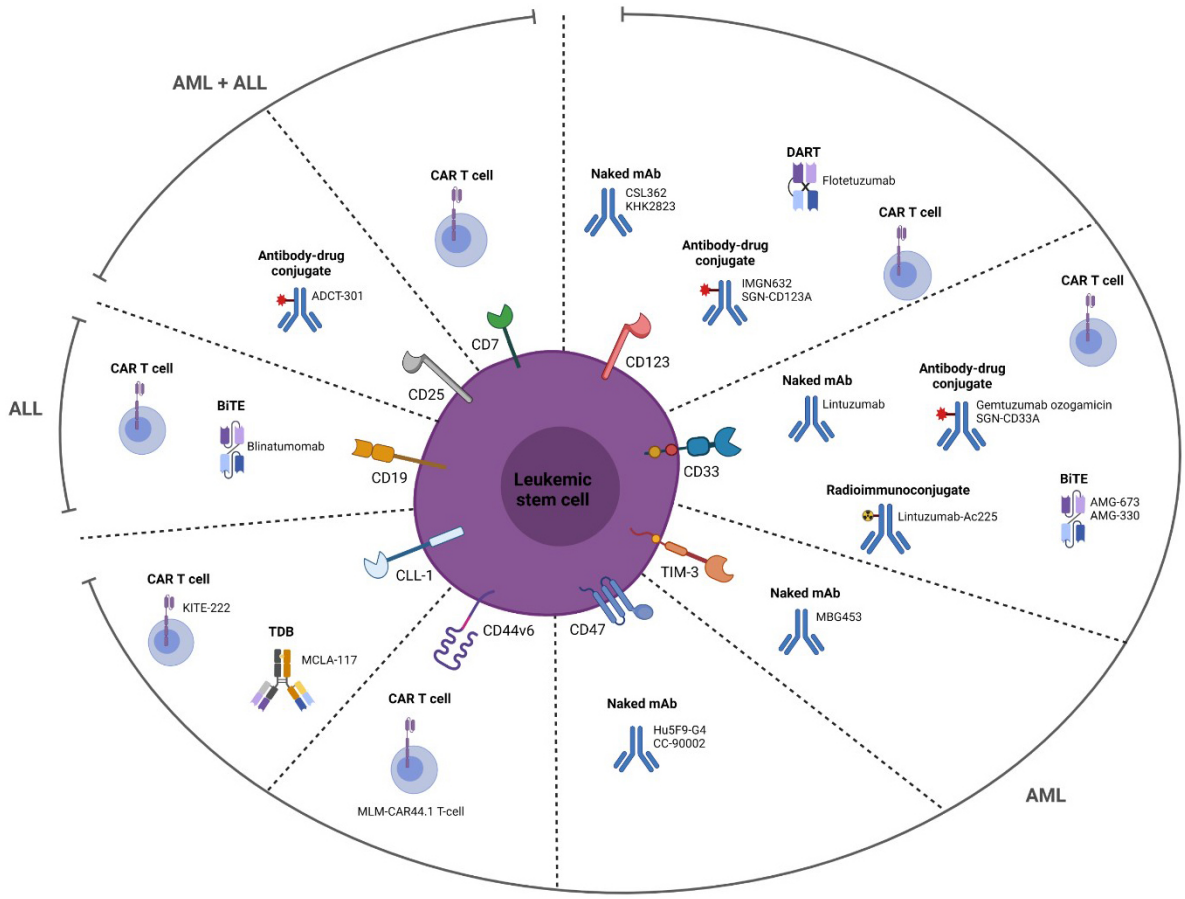


Figure 2. LSC-specific surface markers and the different types of drug agents targeting these markers. All depicted surface markers except CD19 are differentially expressed on AML LSCs. CD19 is a marker specific for B-ALL blasts. In addition, CD7 and CD25 have also been used as targets for ALL treatment. The depicted agents have recently been used in clinical trials. Created with BioRender.com. LSC: Leukemic stem cell; AML: acute myeloid leukemia; CAR: chimeric antigen receptor; TDB: T cell-dependent bispecific; DART: dual-affinity re-targeting antibody.

DISCUSSION

The substantial number of recent studies investigating an LSC population in AML has led to the general acceptance of AML being hierarchically organized. The rare population of LSCs at the apex of this hierarchy plays a key role in the relapse development of the disease due to their drug-resistant mechanisms and self-renewal capacity[17]. Several common surface markers have been identified that enable both the specific eradication and isolation for further analysis of this LSC population [Table 1]. In addition, different pathways regulating LSCs "stemness" are often dysregulated, making them highly promising targets for the elimination of these cells [Table 2]. The focus of current clinical trials is on novel therapies targeting LSC specific markers by using immunotherapy-based drugs such as CAR-T cells directed to LSC specific surface markers or small-molecule inhibitors/agonists directed to "stemness" pathways [Supplementary Table 1]. However, the multiple resistance mechanisms contained by LSCs are a major challenge in achieving an effective eradication of these cells. Therefore, many clinical trials in AML patients test the effectiveness of targeting LSCs using several therapies, both as a monotherapy and in combination with other drugs.

Accumulating evidence shows that the interaction between BM niche and LSCs plays a role in the survival and acquired drug resistance of AML LSCs[177]. For instance, inhibiting the CXCR4/SDF1 interaction

involved in HSC/LSC homing releases LSCs from their chemoprotective niche, making them more sensitive to chemotherapy[30]. This indicates that, besides specifically targeting LSCs, targeting the pathways involved in the BM/LSC interaction could also be a useful strategy to increase LSC eradication. However, this strategy has to be executed carefully, since releasing leukemic cells from the BM into the peripheral circulation could possibly increase the risk of these blasts infiltrating other organs. Future studies on AML should therefore focus on gaining better insight into the factors involved in LSC homing and the effectiveness and safety of targeting these factors.

Compared to AML, research on the LSC population in ALL has been less successful with highly contradicting results. When comparing AML with T-ALL, pathophysiological similarities are seen within both diseases[162,178]. However, it is still not fully understood if T-ALL follows the same stem cell model as AML. The majority of B-ALL cells containing different immunophenotypes have the capacity to initiate ALL, indicating that B-ALL lacks a clear hierarchy as in AML[158]. Hence, the term LSC as used in AML might not be correct to define the cells being able to cause relapse in ALL. For ALL, the term leukemia-initiating cells (LICs) is more preferred. Due to the dynamic phenotypes of LICs, their prospective purification has not been possible up to now[33]. More research is needed to investigate ALL LIC plasticity and discriminating factors that make one population of cells more likely to result in relapse than the other. However, since this has shown to be highly challenging, the focus on interfering with the leukemic cells and BM interaction could be a more promising approach to eliminate ALL LICs in the near future. For all these novel LSC-targeted agents, it is difficult to assess whether specifically the LSC are really eliminated. Monitoring the LSC using flow cytometry during and after therapy might be an option[179,180] but is not standardly investigated yet.

Despite many different surfaces and other markers being promising targets in eradicating LSCs, there are some important considerations that need to be taken into account before applying anti-LSC therapeutics. First, it is extremely important that healthy cells, especially HSCs, are not targeted by the treatment. Since some surface markers and intracellular pathways involved in "stemness" are expressed in LSCs and HSCs, there is a concern that targeting LSCs can harm healthy stem cell populations. This might cause severe and dangerous side effects, so more research is needed to evaluate how many healthy cells are affected in patients treated with these therapies. Secondly, a major challenge is the heterogeneity of acute leukemia. For instance, in AML, heterogeneous phenotypes of LSCs have been identified between patients[181]. In addition, it is possible that there are multiple populations of LSCs with different phenotypes present within one patient. Targeting only one marker may therefore not be effective, and more research focusing on simultaneously or subsequently targeting multiple markers is needed.

DECLARATIONS

Authors' contributions

Planned the conception and design of this review article: Hansen Q, Cloos J
Performed the literature search and wrote the manuscript: Hansen Q
Supervision of the research and editing of the manuscript: Bachas C, Smit L, Cloos J

Availability of data and materials

This review article was based on previously published research and reviews, which were collected by using a search of the literature, and they are cited in the review.

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All authors declared that there are no conflicts of interest.

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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DNA damage and metabolic mechanisms of cancer drug resistance

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Abstract

Cancer drug resistance is one of the main barriers to overcome to ensure durable treatment responses. While many pivotal advances have been made in first combination therapies, then targeted therapies, and now broadening out to immunomodulatory drugs or metabolic targeting compounds, drug resistance is still ultimately universally fatal. In this brief review, we will discuss different strategies that have been used to fight drug resistance from synthetic lethality to tumor microenvironment modulation, focusing on the DNA damage response and tumor metabolism both within tumor cells and their surrounding microenvironment. In this way, with a better understanding of both targetable mutations in combination with the metabolism, smarter drugs may be designed to combat cancer drug resistance.

Keywords: Cancer drug resistance, drug resistance, metabolism, DNA damage, DNA repair, hypoxia, synthetic lethality, overcoming resistance

INTRODUCTION

Over the past decades, our understanding of cancer as a disease has increased immensely. The realization of using DNA damaging agents to inhibit the growth of fast-dividing cells with chemotherapy was a game-changing step in treating many types of cancers[1]. However, as cancer cells are in a more plastic state with increased genomic instability, resistance to single-agent chemotherapy became prevalent. Therefore, following the steps of infectious disease protocols, combination therapies have evolved to combine multiple chemotherapeutic agents to elicit a longer-lasting effect[2]. While this is more beneficial than single-agent treatments, drug/therapy resistance in cancer is still inevitable and universally fatal[3].

Targeted therapies have emerged to combat the rapid drug resistance of broad DNA damaging chemotherapy compounds, which use our increased knowledge of specific vulnerabilities in different types of cancers[4]. Success has been seen targeting specific proteins, such as BCR-ABL, the estrogen receptor[5], the androgen receptor (AR)[6], HER2[7], the epidermal growth factor (EGFR)[8], and others. More recently, targeting the immune system via checkpoint inhibitors, like PD1/PD-L1[9] and CTLA4[10], have produced cures in a subset of patients. Nevertheless, in patients where a cure cannot be achieved with targeted or conventional chemotherapy, cancers will recur and become drug-resistant which ultimately leads to patients' death. Therefore, drug resistance is the principal limiting factor in patient overall survival.

In this review, we discuss relevant resistance mechanisms tumor cells use to adapt to both chemotherapy and targeted therapies. Furthermore, we summarize some of the promising avenues that are currently being investigated to target the tumor resistance pathways and mutations that arise from the treatments. Overall, a better mechanistic insight into drug-resistant cells will hopefully allow for smarter drug design to help combat the major problem of drug resistance and extend patient survival.

MECHANISMS OF RESISTANCE

Changes in the drug-induced DNA damage response

DNA damage continues to be an effective target for cancer therapy as the definition of cancer is uncontrolled cell growth[4]. In this way, increased cell cycling can lead to more error-prone DNA replication, which relies on DNA damage repair pathways to ensure cell fitness. Therefore, targeting DNA replication via chemo- and radio-therapy to induce DNA damage and ultimately cell death is still the most common - and sometimes the most effective - in cancer treatment[11]. However, while chemo- and radio-therapy can be initially successful, therapy resistance in cancer is common and ultimately fatal. For this reason, extensive efforts have been focused on both determining and targeting the protein or pathways involved in chemo- and radio-resistance.

In glioblastoma (GBM) - an extremely deadly brain cancer with a ~14-16-month median survival rate - the standard of care includes radiation therapy (RT), maximal surgical resection, and the chemotherapeutic agent temozolomide (TMZ)[12]. The mechanism of action of TMZ was later shown to create O^6-methylguanine adducts, which would create double-strand breaks (DSBs) post replication. However, TMZ-resistance is rapid and was found to be partly due to the DNA damage repair protein O^6-methylguanine-DNA methyltransferase (MGMT)[13]. MGMT is the suicide DNA repair protein responsible for removing the O^6-methylguanine adducts and allowing for its damage repair over DSB formation and cell death[14]. In this way, MGMT inhibitors have been reported that inhibit the function of MGMT. MGMT inhibition has also been shown, to reverse pancreatic tumor gemcitabine resistance via suppressing the expression of survivin in animal models[15]. However, little clinical success has been realized with MGMT inhibitors with or without TMZ treatment[16].

DNA-protein kinase (DNA-PK) is a DSB DNA damage sensing complex composed of Ku70, Ku80, and the DNA protein kinase catalytic subunit (DNA-PKcs)[17]. After a DSB has occurred, DNA-PK binds to the broken end of the DSB to protect it from nuclease degradation and recruits the other DNA damage repair proteins to initiate DSB repair via non-homologous end joining (NHEJ)[18]. RT has been shown to induce DSBs of which DNA-PK can identify and repair with NHEJ, thereby preventing radiation-induced cell death[19]. Cells which have a decreased expression of DNA-PKcs have also been shown to be more sensitive to RT[20]. In this way, many DNA-PKcs inhibitors have been developed to overcome radiation resistance and/or enhance radiation-induced cell death. Radio-sensitization via DNA-PKcs inhibition has been observed with DNA-PK inhibitor VX-984 in GBM[21], NU7441 in cervical and breast cancers[22], and NU7026 in non-small cell lung cancer[23]. Another DNA-PK inhibitor, NU5455, has also been shown to enhance the killing of doxorubicin in lung cancer models[24].

Lastly, it has been shown that chemotherapy can modulate pro-survival pathways like increasing the expression of drug efflux pumps, apoptosis defects, DNA adduct tolerance, cellular detoxification, and inducing a hypoxic environment[25]. While the metabolic effects of hypoxia will be discussed in more detail below, Chen *et al.*[26] have taken advantage of intracellular hypoxia in cisplatin-resistant cells and created a hypoxia-amplifying DNA repair-inhibiting (HYDRI) nanomedicine. HYDRI specifically targeted cancer cells because of their drug-induced hypoxic environment, and then released its payload of hypoxia-activatable chemotherapeutic tirapazamine. In this way, previous studies which determined upregulated pathways in cisplatin resistance could be used to create a smarter therapy that bypassed drug efflux pumps, induced a unique DNA damage profile, and relied on the inevitable hypoxic environment created by cisplatin resistance to target these drug-refractory models[26].

Synthetic lethality

In 2005, the idea of synthetic lethality in the DNA damage repair pathway, via BRCA1 mutation, was published by two groups[27,28]. Poly ADP ribose polymerase (PARP) 1 is a key activating protein in the single-strand break (SSB) or base excision repair DNA damage repair pathway[29]. In parallel, a double-strand break (DSB) can be fixed by two major DNA damage repair pathways - homologous recombination (HR) or NHEJ. HR utilizes a sister chromatid and has a lower error rate, with BRCA1/2 playing a major activating role for proper DNA damage repair, whereas NHEJ is more error-prone, but quicker in repairing a DSB in interphase[30]. Upon the advent of genetic profiling of tumors, it was discovered that many breast and ovarian cancers had either germline or tumor-specific mutations in the *BRCA1* or *BRCA2* gene[27]. With the knowledge that SSBs that were unresolved by PARP1 became DSBs with replicative stress that requires BRCA1/2 for repair, the idea of cancer DNA damage response (DDR) synthetic lethality was tested[31] [Figure 1]. PARP1 inhibition (PARPi) in BRCA1/2 mutated tumors has been successful in many types of cancers, including breast, ovarian, prostate, pancreatic, colon, and lung[32]. One of the main advantages of tumor specific BRCA mutations is the decreased toxicity of single-agent PARPi treatment. Combination therapies to mimic this synthetic lethality by combining both a BRCA inhibitor with PARPi have proven to be toxic[33].

Currently, there are at least five PARPi - veliparib (Abbvie), rucaparib (Pfizer/Clovis), Olaparib (KuDOS/AstraZeneca), niraparib (Merck/Tesaro), and talazoparib (Lead/Biomarin/Medivation/Pfizer)[32] - where the most common mechanism of action is the "trapping" of PARP on the DNA to induce DSBs[34]. Talazoparib is the newest PARPi and has the highest ability to "trap" PARP on the DNA with ~100 times greater efficacy than the next best PARPi[35]. Nevertheless, this increased trapping of PARP increases the toxicity of talazoparib - compared to other PARPi[36]. In clinical results, the phase 3 trial EMBARCA had 431 BRCA1/2 mutant patients with advanced breast cancer, where the talazoparib group had a 62.6% response rate compared to the 27.2% of the standard chemotherapy group[37].

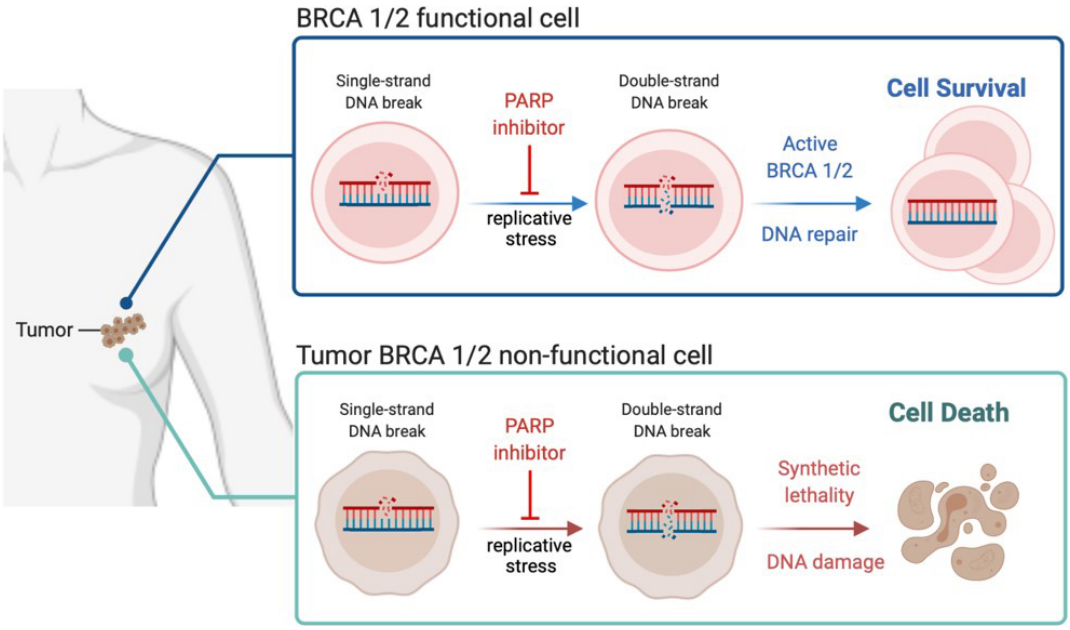

Figure 1. PARPi synthetic lethality in BRCA 1/2 non-functional tumors. BRCA 1/2 functional tumor cells will repair the double-strand break (DSB) induced by PARP inhibition and sequential replicative stress allowing cell survival and growth, whereas BRCA 1/2 non-functional cells cannot repair the DSB and therefore succumb to DNA damage-induced cell death. Figure created with BioRender.

The synthetic lethality success story has other groups actively looking for other synthetic lethal interactions with the many abundant cancer-associated mutations. While not dependent on DDR mutations, AR signaling and PARPi have also been shown to give rise to a synthetic lethal phenotype in preclinical models. However, it is important to note that 19% of primary prostate cancers and 23% of metastatic castration-resistant prostate cancer (mCRPC) have DNA damage repair gene mutations. BRCA2 mutations also have increased levels of prostate specific antigen, a larger percentage of high Gleason scored tumors, and elevated rates of distant and nodal metastases[38]. Clinically, prostate cancer patients treated with abiraterone plus olaparib showed improved radiographic progression free survival over abiraterone alone in a phase 2 trials which was independent of DDR mutations[39]. This has also led to an interest in combining PARPi with androgen deprivation therapy alone or in combination with AR signaling inhibitors, which is currently ongoing in mCRPC, and should be considered even in a non-DDR altered state[38].

DNA damage response with immunotherapy

Immune checkpoint inhibitors (ICI) had great initial promise, with early clinical trial results showing obvious tumor shrinkage, initially. However, after further evaluation, ICI can have about a ~10%-20% durable response rate, depending on the types of cancer[40]. Therefore, like what we previously described with chemotherapy and targeted therapy, combinatorial studies have been designed to determine whether ICI efficacy can be improved when combined with other conventional therapies[41,42].

Melanoma was the first cancer to show preliminary success with ICI, and it is well-known that melanoma has one of the highest rates of tumor mutation burden (TMB)[43]. This brought about the hypothesis that higher rates of TMB in cancer would increase the number of neo-antigens which were predicted to produce a stronger immune response and increase sensitivity to ICI. Radiation and ICI have been tested in combination as radiation treatment for cancer will induce DNA damage, neo-antigens, and immune response[44]. Furthermore, it was shown in 1979 that the effect of radiation is linked to the immune system when twice the dose of radiation was needed to control tumor growth in thymectomized mice compared to

mice with an intact immune system[45]. Within the clinic, overall survival of concurrent radiation and ICI - compared to radiation before or after ICI - was shown to improve overall survival (OS) in a retrospective review of lung cancer patients with distant brain metastases[46].

Other chemotherapy agents are currently being combined with ICI to determine combinatorial efficacy, where one combination - PARPi + ICI - is the most developed. PARPi have shown great promise in many avenues, as shown above with synthetic lethality strategies[47]. With the increasing characterization of PARPi pathway changes, it was noted in breast cancer that PARPi induced PD-L1 expression[48]. Not only has PD-L1 been shown to increase with PARPi, but also an increase in cytoplasmic DNA, which activates the cyclic guanosine monophosphate-AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway[47]. PARPi has also been shown to inactivate the glycogen synthase kinase 2 beta (GSK3)[36]. An *in vivo* model of BRCA-deficient triple-negative breast cancer also demonstrated that PARPi activated the cGAS/STING pathway and increased $CD8^+$ T cell infiltration[48], as well as decreasing T-cell activation resulting in enhanced cancer cell apoptosis[36]. Currently, biomarkers are being investigated to select non-BRCA patients that would respond to PARPi + ICI, where the mutational signature 3 - associated with HR deficiency - positively predicted patient responses[49]. Both the TOPACIO trial and MEDIOLA study are investigating feasibility of immune checkpoint blockade with PARPi[36].

Reactive oxygen species in drug resistance

Reactive oxygen species (ROS) play a well-known role in cell growth and proliferation in cancer cells, where an increase in ROS can enhance cell growth and post-treatment survival[50]. One way in which ROS can increase cancer cell survival is genetically by oxidizing nucleic acids, which will cause random mutations and increase genomic instability[51,52]. ROS can also affect the normal redox balance within the cell. Cysteine is a readily oxidizable amino acid containing a thiol (-SH) group[53]. Many enzymes have active sites that contain necessary cysteine residues to assist in biochemical reactions[54]. In Fms-related receptor tyrosine kinase (FLT)3-ITD (a mutation in the tyrosine kinase domain) expressing acute myeloid leukemia, NADPH oxidase 4 generated ROS will inactivate the protein-tyrosine phosphatase (PTP) DEP-1/PTPRJ, which negatively regulates FLT3-ITD transformation[55]. PTP phosphatase and tensin homolog (PTEN), a PTP family member and potent tumor suppressor, has also been shown to be susceptible to H_2O_2-mediated oxidation and inactivation[56]. As PTEN is a negative regulator of PI3K and Akt pathways, oxidation and inactivation of PTEN augments downstream signaling and cell growth[57].

While ROS have been used to create DNA damage via chemotherapy and are necessary byproducts of radiation, drug-resistant cells have been shown to increase their intracellular ROS levels, and thereby adapt to this intracellular hypoxic environment[58]. Initially, when naïve cells are exposed to chemotherapy, an increase in ROS is noted, as well as a concomitant increase in the antioxidant systems to combat this onslaught of oxidants[58]. However, this seems to differ in some drug-resistant, or persister, cells where an increase in ROS is still true, but antioxidant genes like glutathione peroxidase (GPX) 4 are now downregulated[59]. One hypothesis is that these cells use this ROS to their advantage as the hypoxia response element (HRE) shows a higher binding of HIF1 when the G's of the HRE are modified via ROS[60]. Accordingly, in a new study which dives into the metabolic and transcriptional changes between untreated and persister cells post drug treatment, two of the main upregulated pathways in their model, osimertinib-treated Trp53-knockout with a lung-specific EGFR (L858R) mutation, were ROS and fatty acid metabolism (FAM)[61]. Furthermore, the cells that had the increased ROS/FAM gene signature were also the cycling persister cells, compared with the non-cycling persister cells. In post-treatment patient samples, 8 out of 11 melanoma samples had an increase in either ROS or FAM signatures, as well as 50% of $HER2^+$ breast cancer samples, but the increase in $HER2^+$ samples was only in the post-treatment samples[61].

In these ways, ROS-induced metabolic reprogramming has been an active non-mutational target. The most promising area of research is ferroptosis - or iron-dependent cell death - induction[62]. As drug-resistant cells have been shown to increase ROS and decrease their antioxidant gene expression, this leaves the cells exquisitely sensitive to ferroptosis via GPX4 or xCT inhibition[59]. GPX4 is the main regulator to decrease lipid oxidation, and xCT is a cystine/glutamate antiporter, where cysteine is a necessary component for the reducing agent glutathione[63]. While ferroptosis induction has shown promising initial results in drug-resistant cell and animal models, most drugs never reach the target in cancers such as pancreatic cancer. In this largely drug-refractory cancer, Badgley *et al.*[64] showed that a cyst(e)ine degrading enzyme - cyst(e)inase - was able to deplete cyst(e)ine from the extracellular environment, thereby decreasing intracellular cyst(e)ine and preventing GSH production. In both *in vitro* and *in vivo* models, cyst(e)ine deprivation led to robust induction of ferroptosis, cell death, and longer animal survivals[64].

Drug resistance via lipid metabolism and import

Fatty acid (FA) metabolism plays a host of roles within the cell. FAs may be most well-known for being membrane building blocks with the synthesis of glycerophospholipids[65]. Interestingly, the lipid composition of membranes has garnered recent interest as chemotherapy-resistant cancer cells, in preclinical models, show reduced fluidity of their membranes. These membranes have an increase in saturated fatty acyl chains and are especially enriched for monounsaturated fatty acyl chains in glycerophospholipids[66]. While this may seem inconsequential, one of the most promising therapeutic targets of drug-resistant cells - ferroptosis - can depend on poly-unsaturated fatty acyl chains to induce toxic lipid peroxidation and cell death[67]. Furthermore, in chemotherapy-resistant leukemia or ovarian cancer cell lines, the reduced membrane fluidity can come from an increase of cholesterol and/or sphingomyelin within the membrane[68,69]. This stiffened membrane has been shown to decrease passive diffusion of drug uptake and enhance detergent-resistant membrane domains, which can activate the family of ATP-binding cassette multidrug efflux transporters - including p-glycoprotein - potentiating the multidrug-resistant (MDR) phenotype. However, modulation of membrane fluidity was able to alter the drug efflux transporters, suggesting a potential for diet interventions[70].

Targeting lipid synthesis itself may also have a benefit in re-sensitizing cells to chemotherapy. Fatty acid synthase (FAS) inhibitors have re-sensitized ovarian cells *in vitro*[71,72], *ex vivo*[71], and *in vivo* for T cell lymphoma and ovarian cancer models[73,74]. In breast cancer cells, overexpression of FAS was able to confer chemoresistance *in vitro*[72]. While the mechanism by which FAS inhibition can alter chemotherapy sensitivity is unknown, a decrease in MDR proteins has been observed, suggesting membrane composition may be important[75]. Fatty acid oxidation has also been shown to increase with chemotherapy resistance. GBM cellular and patient-derived xenograft models showed an increase in fatty acid beta-oxidation post-TMZ treatment[76]. In breast cancer patient samples, the necessary beta-oxidation enzyme carnitine palmitoyltransferase I (CPT1) was increased in tumors that recurred and was also higher in chemo-resistant tumors[77]. CPT1 inhibitors have also been shown to re-sensitize tumor cells to chemotherapeutic agents[78].

Lastly, lipid droplet (LD) number and function play an important role in chemotherapy resistance. LDs may directly assist in cell survival by providing an energy reserve of lipids to be oxidized in case of nutrient deprivation[79]. Hydrophobic drugs can also be sequestered within lipid droplets, creating a drug "sink" for detoxifying chemotherapeutic agents[79]. Interestingly, LDs were found to co-localize with the mitochondria more frequently in chemo-resistant breast cancer cells, where the LD protein perilipin 4 (PLIN4) was increased. PLIN4 assists in mobilizing lipids for oxidation from LDs, where silencing of PLIN4 decreased the growth of the chemotherapy-resistant, but not the parental, breast cancer cells, suggesting that lipid beta-oxidation is necessary for the sustained growth of chemotherapy-resistant cells[80] [Figure 2]. Inhibition of long-chain fatty acyl-CoA synthetase via triacsin C blocked fatty acid activation and LD biogenesis which

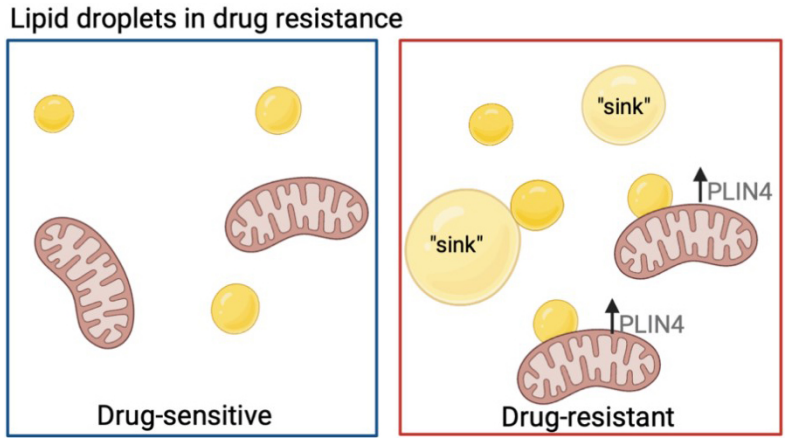

Figure 2. Lipid droplet usage in drug-resistant cells. Drug-resistant cells have been shown to increase lipid droplet accumulation (yellow circles) and have a higher percentage co-localized to the mitochondria, where an increase in PLIN4 helps to better utilize lipids for fatty acid beta-oxidation. Lipids can also be used as drug "sinks" for hydrophobic drugs. Figure created with BioRender.

rendered drug-resistant colorectal cancer cells sensitive to chemotherapy treatment both *in vitro* and *in vivo*[81].

Hypoxia and drug resistance

The 2019 Nobel Prize was awarded to Kaelin, Ratcliffe, and Semenza for their seminal work on discovering how oxygen is sensed in the cell and the way in which cells are able to adapt to changing oxygen concentrations[82,83]. Hypoxia-inducible factor (HIF) and HIF signaling are now realized as both a hallmark of cancer, and also affect the surrounding microenvironment[4]. Through large data analyses, Bhandari *et al.*[84] used the Buffa-defined hypoxia signature to determine the breadth of hypoxia amongst 1188 samples from 27 types of cancer. They found that hypoxia was varied both between cancers and even within a single patient. The most hypoxic tumors were cervical squamous cell carcinoma and lung cancer, with thyroid adenocarcinoma and chronic lymphocytic leukemia being the least hypoxic[84]. This study corroborated the findings of The Cancer Genome Atlas. Furthermore, higher hypoxia scores also correlate to both lower overall survival and progression-free survival in multiple cancer types[85]. A forced hypoxic environment in GBM models has also been shown to decrease the sensitivity of cells to the standard of care chemotherapeutic agent TMZ[86].

As cancer cells grow faster than normal cells, they quickly outgrow their nutrient supply, which creates a lower level of oxygen in the tumor microenvironment (TME). This low oxygen, or hypoxic TME, affects gene signatures and pathways activated within the tumor cells[87]. Carbonic anhydrases and CO_2 levels are increased, which leads to increased cellular acidification[88]. However, cancer cells depend on a higher intracellular pH (~7.4 *vs.* ~7.2) and acidify their TME, decreasing the extracellular pH (~6.0-~7.1 *vs.* ~7.4)[89]. This acidic niche has been shown to increase the expression of MDR genes and decrease drug import into cancer cells[90]. A highly acidic TME can also prevent proper immune profusion and cause resistance to ICI[91]. Acidity can increase the immune checkpoint protein expression, CTLA-4 on T cells, raise the threshold for T cell activation[92], and decrease $CD8^+$ memory T cell lifespan[93]. Long-term exposure to an acidic environment can decrease natural killer cell function, activation, and survival[92]. Therefore, modulation of TME pH has been an active area of research.

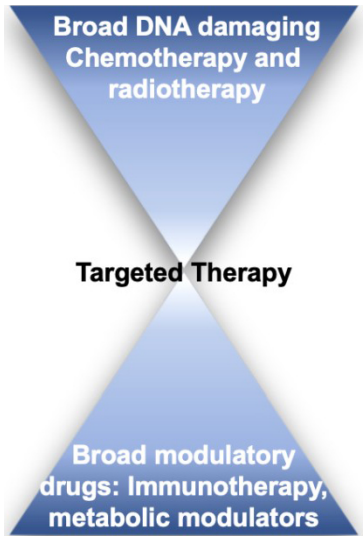

Figure 3. Therapy schematic. Broad-reaching drugs may lend to more durable responses as resistance can arise more rapidly to targeted therapy. Modulating more broad cancer hallmarks - like immune and metabolic targets - may offer smarter drug targets.

To neutralize the acidic TME, oral bicarbonate was given and artificially increased the pH of the TME, which allowed for a better response to ICI in multiple cancer models[94]. In an effort to maintain normal levels of pH, Na^+/H^+ exchanges, like sodium-hydrogen exchanger isoform (NHE), are upregulated to uptake sodium and pump out protons[95]. The Na^+/H^+ exchangers are the most common membrane proteins and attempt to regulate the hypoxia-induced pH changes within the cell[96]. As intracellular acidosis can induce necroptosis and apoptosis, NHE1 inhibitors have been found to modulate intracellular pH and lead to cell death[97]. Cariporide, an NHE1 inhibitor, can induce apoptosis in breast cancer, reduce MDR1 expression, and decrease tumor volume[98]. Another NHE inhibitor, amiloride, increased ROS abundance, thereby stimulating PAR synthesis and inducing the PAR-dependent cell death termed parthanatos[99]. In GBM, Na^+ was shown to be increased almost 3-fold between cancer and normal cells, while NHE1 overexpression is noted and increased NHE1 correlated to worse overall survival[97].

CONCLUSIONS AND FUTURE PERSPECTIVES

Cancer drug resistance remains the biggest challenge in successfully treating cancer patients today. Here we have outlined some of the current strategies to target the DNA damage repair proteins via chemo-induced dependencies, synthetic lethality, and combination with immunotherapy. More research insight into cancer-specific deficiencies can only lead to better responses, or third-line therapeutic options. Tumor metabolism and the microenvironment may also prove to be promising drug targets as uncontrolled cell growth will always be a hallmark of cancer. Therefore, if we are truly able to understand the metabolic changes and vulnerabilities of cancer, we may be able to develop biomarkers to help dictate metabolic status and treatment plans.

As a whole, we may be better off looking at broad regulatory pathways, as we seem to be at the far end of an hourglass curve [Figure 3]. In the beginning, broad DNA damaging agents were game-changing as they targeted the quintessential cancer dependency - cell growth. Then we narrowed the focus to specific proteins with either activating mutations or cancer-driving functions. While some success has been achieved with targeted therapy, new avenues like modulating the immune system, or using HYDRI-like methods to target broad cancer hallmarks may lead to more durable and smart therapeutic designs.

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Authors' contributions

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Wrote the manuscript: Tiek D
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Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Venetoclax resistance: mechanistic insights and future strategies

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Abstract

Acute myeloid leukemia (AML) is historically associated with poor prognosis, especially in older AML patients unfit for intensive chemotherapy. The development of Venetoclax, a potent oral BH3 (BCL-2 homology domain 3) mimetic, has transformed the AML treatment. However, the short duration of response and development of resistance remain major concerns. Understanding mechanisms of resistance is pivotal to devising new strategies and designing rational drug combination regimens. In this review, we will provide a comprehensive summary of the known mechanisms of resistance to Venetoclax and discuss Venetoclax-based combination therapies. Key contributing factors to Venetoclax resistance include dependencies on alternative anti-apoptotic BCL-2 family proteins and selection of the activating kinase mutations. Mutational landscape governing response to Venetoclax and strategic approaches developed considering current knowledge of mechanisms of resistance will be addressed.

Keywords: Venetoclax, acute myeloid leukemia, hypomethylating agents, Azacitidine, Decitabine, resistance, BCL2 protein, human

INTRODUCTION

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults[1]. The disease often affects older adults with a median age of 68 years at diagnosis[1]. Conventional treatments using intensive chemotherapy are less beneficial for older patients with poor tolerance and modest outcome[2,3].

Introduction of Venetoclax (ABT-199), a selective inhibitor of BCL-2, has advanced the treatment options for AML patients, especially older patients[4]. Venetoclax in combination with hypomethylating agents (HMA) or low-dose cytarabine (LDAC) was approved by US FDA for newly diagnosed AML patients who are either unable to receive intensive chemotherapy or older than 75 years old. Nowadays, this has become the standard therapy for such a population[5]. However, the prevalent use of Venetoclax comes with a new challenge of resistance, particularly in the relapsed/refractory setting[6,7]. Here, we review the mechanisms of Venetoclax resistance in AML and discuss strategies to overcome resistance.

MECHANISM OF ACTION OF VENETOCLAX

BCL-2 family proteins and mechanism of action of Venetoclax

Venetoclax (ABT-199) is an oral, selective antagonist of the B-cell lymphoma 2 (BCL-2), a key protein modulating intrinsic (mitochondrial) apoptosis[8]. Apoptosis is regulated and balanced by protein-protein interactions among BCL-2 family members[9]. Different members of the BCL-2 family share BCL-2 homology motifs (BH1 to BH4)[9]. Anti-apoptotic proteins (BCL-2, BCL2A2, MCL-1, and BCL2L1 (BCL-xL), BCL-w, BFL-1/A1) sequester pro-apoptotic proteins by binding to its BH3 motifs[9-13]. Pro-apoptotic proteins consist of BH3-only proteins and effector proteins, BAK and BAX, which have BH1-4 motifs. BH3-only proteins act as sensitizers (BAD, BIK, HRK, NOXA) or activators (BIM, BID, PUMA) of apoptosis[10,11,13]. BH3-only sensitizer proteins are unable to activate downstream effector proteins (BAX, BAK) directly. However, they are able to "sensitize" cells toward apoptosis by binding to BCL-2 anti-apoptotic protein, releasing bound BAX or BAK or BH3-only activator protein[14]. Upon activation by bound BH3-only activator proteins, effector protein oligomerizes, leading to increase mitochondrial outer membrane permeabilization (MOMP) and initiation of cytochrome *c* mediated intrinsic apoptosis[9-13].

Dysregulation and imbalance of BCL-2 family members controlling apoptosis are commonly found in multiple hematologic malignancies[12]. Likewise, the BCL-2 family has an essential role in mediating AML survival and chemoresistance. Previous studies have demonstrated that an increase in the level of anti-apoptotic proteins, including BCL-2, is associated with chemotherapy resistance[15-17]. BCL-2 also supports the survival of leukemic stem cells in AML, and its inhibition induces the death of quiescent leukemic stem cells[18]. Hence, BCL-2 is an important therapeutic target. Venetoclax, a BH3 mimetic, binds to a BH3-binding groove of BCL-2 protein with high selectivity[8]. This relieves inhibition of BCL-2 toward BAX and BAK, resulting in cell death[9]. Preclinical studies demonstrated high anti-tumor activity of Venetoclax in AML, which facilitated further clinical studies[16,19]. In an initial phase 2 study, Venetoclax monotherapy produced modest responses in heavily treated relapsed or refractory (R/R) AML patients with an overall response rate (ORR) of 19%[4]. As AML may not depend on BCL-2 for its survival or the dependency may evolve during tumor progression and after therapy[20], a method to assess BCL-2 family dependency is critically needed to predict sensitivity to Venetoclax[4,13,21,22]. BH3 profiling is performed by exposing mitochondria to a specific BH3 peptide, followed by measurement of cytochrome *c* release and MOMP[23]. Addiction to a specific BCL-2 anti-apoptotic protein is inferred from cellular apoptotic sensitivity when exposed to different BH3 peptides[23].

Genomic biomarkers associated with Venetoclax sensitivity

Early phase 2 study on Venetoclax monotherapy suggested spliceosomal mutation in SRSF2/ZRS2 and IDH1/2 as predictors for Venetoclax sensitivity[4]. Ten out of 11 patients with baseline SRSF2 or ZRSR2 mutation had a measurable reduction in bone marrow (BM) blast after treatment with Venetoclax[24]. *SRSF2* genes were found to induce alternative splicing of apoptosis regulating genes and modulate the expression of BCL-2 family proteins, which may increase sensitivity to Venetoclax[24,25].

In preclinical models, IDH mutation in AML conferred high BCL-2 dependence and sensitivity to BCL-2 inhibition. The mechanism involved oncometabolite (R)-2 hydroxyglutarate (2-HG), which inhibits mitochondrial cytochrome *c* oxidase, causing a reduction of mitochondrial threshold for apoptosis induction[26]. A recent study by Stuani *et al.*[27] described additional mechanisms behind IDH mutant sensitivity to BCL-2 inhibitor through inhibition of mitochondrial respiration. Mutant IDH cells and patient-derived xenografts (PDX) were found to have a larger capacity for mitochondrial oxidative phosphorylation (OXPHOS), as evident by increased electron transport chain (ETC) complex I activity, NADH production by tricarboxylic acid cycle enzymes, mitochondrial ATP content and oxygen consumption rate[27]. Gene set enrichment analysis on IDH1 mutant cells also showed enhanced fatty acid oxidation (FAO) gene signature, especially CPT1a, an acyl-carnitine transporter during FAO, and its transcriptional regulator, CEBPα[27]. While 2-HG was shown to drive CPT1a and CEBPα-dependent FAO and OXPHOS, abrogation of 2-HG production by IDH inhibitor did not impact FAO rate or OXPHOS in the treated AML cell lines, suggesting maintenance of OXPHOS phenotype independent of 2-HG[27]. Inhibition of OXPHOS and IDH1 mutation subsequently showed synergistic cytotoxic activity and improved cell differentiation *in vitro* and *in vivo*[27].

Venetoclax, a BCL-2 inhibitor, was reported to target leukemia stem cells (LSCs) metabolism through reduction of ETC complex II activity and OXPHOS[28]. Consistent with preclinical data, IDH mutant cells showed heightened sensitivity upon OXPHOS inhibition by Venetoclax therapy[27]. In the phase 2 trial of a single agent Venetoclax, IDH1/2 mutated R/R AML subset reached a higher objective response rate of 33% as compared to 10% ORR in the IDH-wildtype AML subset[4]. Mirroring synergy seen in preclinical study[27], a preliminary analysis from an ongoing phase 1/2 clinical trial demonstrated encouraging composite complete remission of 75% with median overall survival (OS) of 9.7 months in the R/R cohort upon treatment with a combination of Ivosidenib (an IDH1 inhibitor), Venetoclax and Azacitidine "triplet"[29]. Future updates are anticipated to evaluate clinical characteristics and molecular predictors of response. In parallel, a phase 1b/2 study of Enasidenib (an IDH2 inhibitor) and Venetoclax combination is underway (NCT04092179) after demonstrated efficacy in preclinical studies[30,31].

Utilizing highly sensitive quantitative reverse transcription polymerase chain reaction (RT-qPCR), a clinical study by DiNardo *et al.*[32] found a strikingly high rate of mutation clearance in NPM1 mutated patients upon treatment with a combination of Venetoclax and HMA or LDAC (4/4 cases). As expected, a high molecular remission rate translated into an excellent survival with relapse-free survival not reached after a median follow-up of 20 months in patients who initially had persistent mutation despite intensive chemotherapy[33]. Unlike IDH mutated AML, the mechanistic basis of Venetoclax sensitivity in NPM1 mutated AML is not well understood[32]. However, clinical findings indicate NPM1 (without FLT3 co-mutation) as an important predictor of Venetoclax sensitivity.

ASXL1 mutation was recently found to drive response to Venetoclax *in vitro*. ASXL1 acts as an epigenetic regulator via PRC2-mediated chromatin modification to keep various genes in a repressed state[34]. Thus, ASXL1 mutation leads to aberrant activation of its target genes, including BCL2[34]. In preclinical study of Rahmani *et al.*[34], ATAC-sequencing analysis suggested higher chromatin accessibility on the BCL-2 locus of ASXL1 mutant KBM5 cells due to failure of PRC2 complex recruitment, resulting in BCL-2 overexpression[34]. BH3 profiling further showed higher BCL2 anti-apoptotic protein dependency in ASXL1 mutant cells, which explained ASXL1 sensitivity to Venetoclax *in vitro*[34]. Rahmani *et al.*[34] also demonstrated enhanced global cytosine methylation, which led to sensitivity to Azacitidine, a DNMT inhibitor. Thus, treatment of ASXL1 mutant cells with single agent Venetoclax or Azacitidine resulted in increased cell differentiation, decreased cell growth and viability[34]. Analysis of data from clinical trials is

needed to prove this finding in the clinical setting.

VENETOCLAX RESISTANCE: PRECLINICAL STUDIES

Cellular mechanism of resistance to Venetoclax: other BCL-2 family proteins

Dependencies on other anti-apoptotic BCL-2 family members, including BCL2-A1, MCL-1, and BCL-xL, have been demonstrated as the key contributors to primary or adaptive Venetoclax resistance[4,7,13,35-37]. Venetoclax resistance is associated with sequestration of BIM freed from Venetoclax binding to BCL-2 by other Bcl-2 family members, which consequently prevents apoptosis [Figure 1][38,39]. Preclinical work demonstrated lower expression of MCL-1 and BCL-xL in Venetoclax sensitive AML cell lines and lower BCL-2 protein levels in resistant cells[13]. When resistance was induced in AML cell lines through exposure to Venetoclax over several months, a shift was seen toward upregulation of MCL-1 and or BCL-xL with less dependency on BCL-2[35,40]. To investigate this potential mechanism, BCL-xL and MCL-1 were inactivated with BCL-xL inhibitor (WEHI-539) and short hairpin RNA (shRNA), respectively, which resulted in the restoration of Venetoclax sensitivity in resistant AML cell lines[35]. Utilizing BH3 profiling, a phase 2 study of Venetoclax monotherapy for R/R AML showed shorter durability of Venetoclax responses in patients with MCL-1 or BCL-xL dependence, suggesting that these mechanisms are operational in the clinical setting[4].

The knowledge around common co-dependencies in leukemia and cancer led to the exploration of selective BCL-xL and MCL-1 inhibitors. Navitoclax (ABT263), a predecessor of Venetoclax, possessed a high affinity for BCL-2, BCL-xL and BCL-w[41]. However, its clinical use has been limited by on-target toxicity of thrombocytopenia due to reliance on BCL-xl for platelet survival[42]. Recently there is a growing interest in the exploration of MCL-1 inhibitors, which have shown striking synergy with BCL-2 inhibitors in resistant AML cell lines and xenograft models[40,43,44]. Multiple MCL-1 inhibitors are under development, and several are being evaluated in clinical trials[45-47].

In recent studies, transcriptomic analysis of AML patients' samples showed differential expression of BCL2A1 in resistant cells[36,37]. Upregulation of BCL2A1 expression correlated with higher Venetoclax's area under the curve (AUC) and less apoptosis after treatment with Venetoclax with or without Azacitidine and Cytarabine. Knockdown of BCL2A1 restored apoptosis and reduced cell growth in the resistant AML cells without substantial effect on the CD34+ hematopoietic stem and progenitor cell (HSPC)[37]. Therefore, there is a potential for synergy between Venetoclax and BCL2A1 inhibitor in selective AML subsets, similar to Venetoclax and MCL-1 inhibitor[37,44].

Mutations in activating kinases and Venetoclax resistance

Activation of intracellular signaling pathways by KRAS/PTPN1 or FMS-like tyrosine kinase 3 (FLT3) mutant proteins is postulated to induce Venetoclax resistance. Genomic biomarkers were analyzed before and after treatment with Venetoclax monotherapy[24]. Three out of 14 patients with pre-treatment FLT3-internal tandem duplication (ITD) and 4 out of 14 patients with PTPN11 mutation failed to achieve bone marrow blast reduction, suggesting intrinsic resistance to Venetoclax. In addition, a subset of patients at the time of relapse were found to harbor FLT3-ITD and/or PTPN11 mutations not identified prior to therapy, strongly indicating the emergence or selection of these mutations as secondary or acquired resistance[24].

FLT3-ITD mutation occurs in about 25% of adult AML cases and confers an adverse prognosis[27]. FLT3-ITD mutation promotes survival via activation of PI3K-protein kinase B (Akt), RAS-MAPK, and STAT5 pathway[14,38]. While the precise downstream molecular pathways are yet to be defined, FLT3-ITD mutation is known to induce higher expression of BCL-xL and MCL-1, which may contribute to Venetoclax

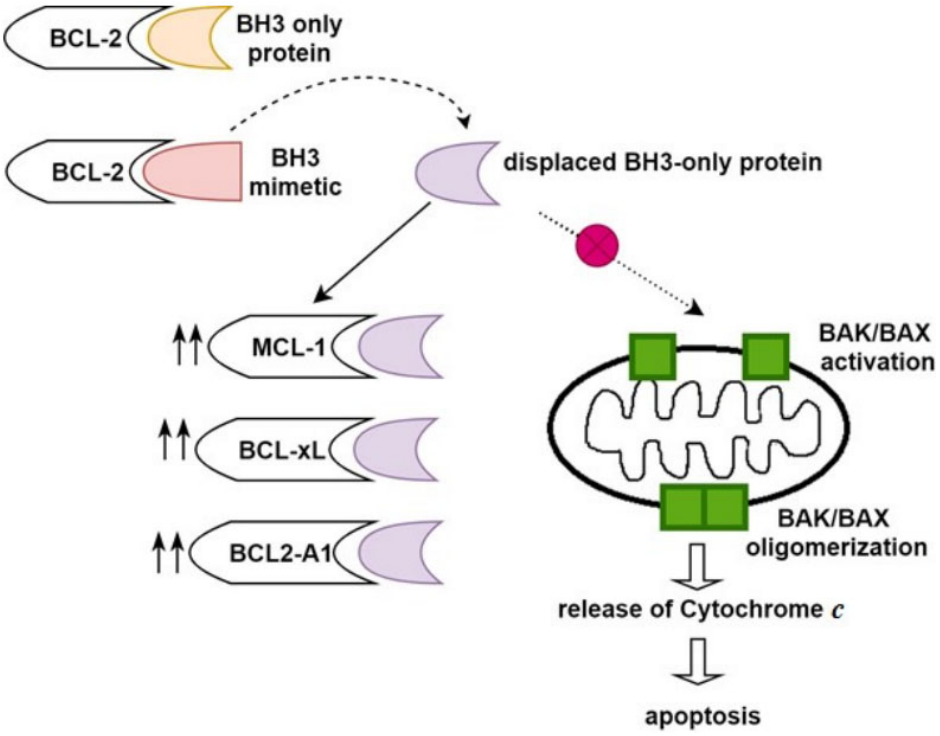

Figure 1. Binding of BH3 mimetic, Venetoclax to BCL-2 anti-apoptotic protein releases bound BH3-only protein, subsequently allowing interaction between BH3-only protein and BAK/BAX. Upregulation of MCL-1, BCL-xL, and BCL2-A1 confers Venetoclax resistance by sequestration of BH3 only proteins, preventing them from interacting with BAK/BAX and avoidance of apoptosis[35-40].

resistance [Figure 2][48-50]. Several studies demonstrated the involvement of the molecular pathway downstream of FLT3 in modulating BCL-xL and MCL-1 expression. STAT5, which was activated by FLT3-ITD, but not by their wild-type counterpart[50], was found to regulate transcription of the *BCL-xL* gene[51]. Akt, a downstream substance of PI3K pathway, was shown to influence MCL-1 stabilization by inactivation of glycogen synthase kinase 3 (GSK3), leading to sequestration of BIM and prevention of MOMP[52]. Yoshimoto *et al.*[50] further reported the role of STAT5 and Akt in MCL-1 upregulation. Suppression of STAT5 by small interfering RNA (siRNA) reduced the level of MCL-1 protein and mRNA in FLT3-ITD$^+$ MV4-11 cell lines. In addition to direct stimulation of MCL-1 transcription, STAT5 also enhances phosphorylation of Akt, which indirectly increases MCL-1 expression in FLT3-ITD cells[50].

Several preclinical studies evaluated the efficacy of combining FLT3 inhibitor and Venetoclax in FLT3-ITD$^+$ cell lines, patient samples, and xenograft models. Ma *et al.*[38] assessed the efficacy of Midostaurin (1st generation type 1 FLT3 inhibitor)[3] and Gilteritinib (2nd generation type 1 FLT3 inhibitor)[3] in combination with Venetoclax. Midostaurin or Gilteritinib in combination with Venetoclax synergistically induced apoptosis in FLT3-ITD+ cell lines and patient samples[38]. The combination of Gilteritinib and Venetoclax also resulted in higher survival of the MV4-11 xenograft model compared to either drug alone, with four out of five treated mice remaining disease-free on day 190[38]. A similar finding was reported by Zhu *et al.*[14] The combination of Gilteritinib with Venetoclax outperformed their respective monotherapy in halting the proliferation of FLT3-ITD$^+$ cells and reducing tumor burden in the FLT3-ITD$^+$ patient-derived xenograft (PDX) and resistant MOLM-14 model[14]. Another set of experiments with a combination of 2nd generation type 2 FLT-3 inhibitor (Quizartinib)[3] and Venetoclax *in vitro* and *in vivo*, resonated with prior studies[49]. Co-treatment with Quizartinib and Venetoclax in the xenograft model produced a longer response with a delay in tumor resurgence for up to three months post-treatment[24].

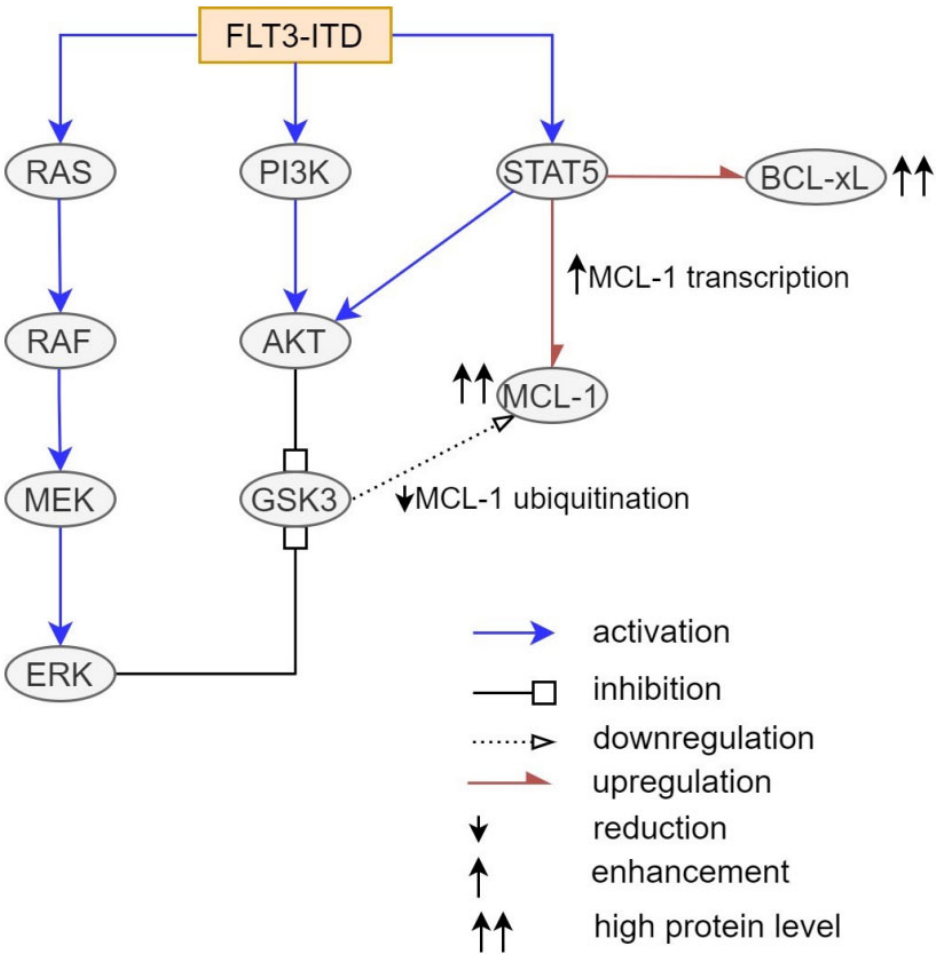

Figure 2. FLT3-ITD mutation causes an increased level of BCL-xL and MCL-1 via activation of downstream PI3K-AKT, RAS-MAPK, and STAT5 pathways. AKT and ERK promoted inhibitory phosphorylation of GSK3, leading to a reduction of MCL1 ubiquitination and degradation. In addition to upregulation of MCL-1 and BCL-xL, STAT5 also increases MCL-1 indirectly through AKT activation. In summary, FLT3-ITD mutation confers Venetoclax resistance by upregulation of BCL-xL and MCL-1[48-53].

In these studies, mechanisms driving the synergy between FLT3 inhibitor and Venetoclax were interrogated. Ma *et al.*[38] and Zhu *et al.*[14] demonstrated that treatment with FLT3 inhibitor (Midostaurin or Gilteritinib) alone or in combination with Venetoclax reduced the expression of MCL-1 *in vitro*[14,38]. Utilizing western blot, reduced phosphorylation of ERK, AKT, STAT5 was seen after 24 hours of treatment with Gilteritinib or Quizartinib. This finding suggests FLT3 inhibition modulates MCL-1 by simultaneous suppression of multiple kinase pathways including RAS-MAPK, PI3K-AKT, and STAT5[14,38]. Co-immunoprecipitation assay in FLT-ITD$^+$ cell lines further revealed decreased binding of BIM to MCL-1 and increased binding of BIM to BCL-2 after Gilteritinib treatment, while the opposite was seen with Venetoclax treatment[14,38]. Interestingly, co-treatment with Gilteritinib and Venetoclax also increased the binding of BIM to BAX without increasing BIM binding to other BCL-2 anti-apoptotic proteins, specifically BCL-xL[14]. Thus, combination therapy with FLT3 inhibitor and Venetoclax *in vitro* reduced BIM binding to both BCL-2 and MCL-1, liberating BIM to interact with BAX and induce apoptosis[14].

In addition to FLT3, mutations in other activating kinases can confer resistance to Venetoclax (so-called "signaling" mutations). Genomic data from primary patient samples in the BEAT AML database was analyzed along with Venetoclax AUC established *in vitro*[37]. Samples with KRAS or PTPN11 mutations

were found to have higher Venetoclax AUC. Venetoclax resistance was reproduced when AML cell lines were transduced to overexpress G12D KRAS and A72D PTPN11[37]. Analysis with RT-PCR and immunoblot showed decreased BCL-2 and BAX with increased MCL-1 and BCL2A1 levels in G12D KRAS cells, as well as increased MCL-1 and BCL-xL levels in A72D PTPN11 cells [Figure 3][37]. G12D KRAS cells showed a reduction in cellular viability after treatment with MCL-1 inhibitors (AZD5991)[37]. However, neither BCL-2 blockade nor simultaneous blockade of BCL-2, BCL-w, and BCL-xL by ABT263 and ABT737 produced a similar response[37]. This finding implied KRAS mutation dependency on MCL-1 to drive Venetoclax resistance. In A72D PTPN11, AZD5991 was also capable of suppressing PTPN mutant cells, while partial response only was seen with BCL-2/Bcl-XL dual inhibitors ABT-263 and ABT 737. This finding suggests partial dependence of PTPN-induced Venetoclax resistance on MCL-1 and BCL-xL[37]. As expected, the combination of AZD5991 and Venetoclax showed synergy and fully rescued mutant cell lines from KRAS- and PTPN11-mediated resistance. Hence, combinatorial therapy with Venetoclax and MCL-1 inhibitor is expected to demonstrate high efficacy in AML patients harboring these mutations[37].

Roles of TP53, BAX, and mitochondria in Venetoclax resistance

In a recent report, BAX variants were found by deep sequencing performed on samples derived from AML patients who relapsed after initially achieving remission with Venetoclax-based regimens, signifying acquired BAX mutation as adaptive Venetoclax resistance[54]. Reduced survival was also seen when BAX deficient OCI-AML3 cells were transplanted into the AML xenograft model[54]. BAX deficient cells and xenograft model were resistant to cell death induced by Venetoclax, MCL-1 inhibitor (S63845), or a combination of both[54]. This is contrary to a prior study which showed sensitivity of BAX knockout (KO) cells to a different MCL-1 inhibitor (AZD-5991) with similar resistance to Venetoclax and BCL-2/BCL-xL inhibitor (AZD-4320)[55]. Hence, particular attention to BAX mutant subsets is warranted in future studies to evaluate its impact on response to BH3 mimetics as single agents or combinations.

Genome-scale CRISPR/Cas9 screening identified BAX along with TP53 and PMA1P1 (NOXA) as genes whose inactivation confers Venetoclax resistance[40,55,56]. Gene enrichment and protein-protein interaction analysis identified these genes as an essential part of the mitochondrial apoptosis pathway[55,56]. TP53 is activated by cellular stress and functions as a transcription factor for genes controlling various cellular processes, including apoptosis and cell cycle arrest[55]. Several BH3-only proteins, including *BAK, BAX, PUMA,* and *NOXA*, are also TP53 target genes[55,57,58]. As expected, a lower level of BAK1, PUMA, and NOXA was observed in TP53 KO cells[55]. Interestingly, transcriptional changes were observed outside TP53 target genes with an increased ratio of BCL-xL/BCL-2, which may further confer Venetoclax resistance in TP53 KO cells[55].

Several preclinical studies demonstrated that TP53 mutated cells and xenograft models are resistant to single-agent therapy with Venetoclax[55,59] or MCL-1 inhibitor[55,59,60]. Contesting these findings, Thijssen *et al.*[60] reported observation that the lack of TP53 function did not preclude cell killing by sub-lethal BCL-2 or MCL-1 inhibitor monotherapy. However, this response was not durable as surviving TP53 deficient cells outgrew TP53 wild-type cells over a longer period of exposure, indicating a competitive survival advantage[60]. Delayed activation of BAX/BAK and subsequent apoptosis upon BH3 mimetics treatment was thought to cause reduced BH3 mimetics efficacy by lifting the early apoptosis threshold in the absence of TP53. Notably, simultaneous inhibition of BCL-2 and MCL-1 was able to overcome the apoptotic delay with improved durability of response[60], resonating with synergistic action described in past studies[55,59].

The impact of TP53 and BAX on mitochondrial function and morphology was evaluated. TP53 and BAX KO cells demonstrated less mitophagy when exposed to stress by a mitochondrial uncoupler and increased

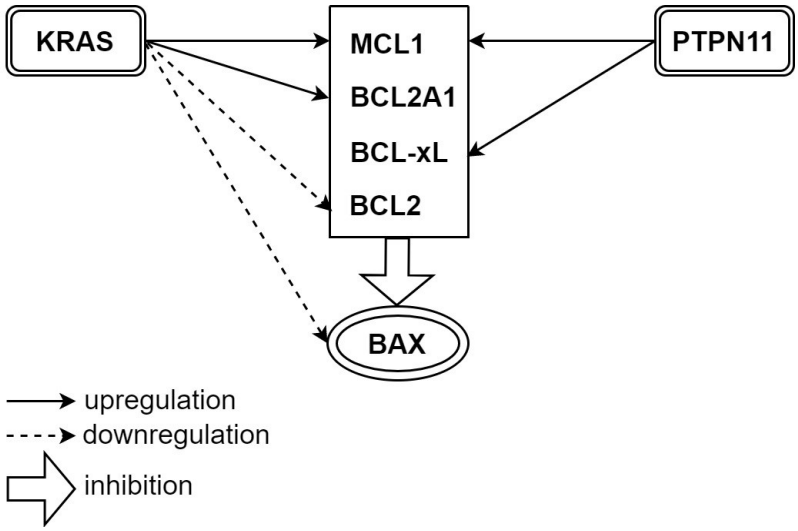

Figure 3. Both KRAS and PTPN11 mutations confer Venetoclax resistance. KRAS mutation causes upregulation of MCL-1 and BCL2A1, while PTPN11 mutation causes upregulation of MCL-1 and BCL-xL. KRAS mutation also downregulates BCL-2 and BAX[37].

cellular respiration, reflected by higher production of cellular reactive oxygen species (ROS)[55]. Furthermore, TP53 and BAX KO cells also showed aberrant metabolic profiles with increased nucleotide synthesis and parallel decreases in glucose, pyruvate, amino acids, and urea cycle intermediates levels, suggesting priority shifting on carbon usage to support cancer cell proliferation[55]. These findings highlight the crucial role of mitochondria in mediating sensitivity or resistance to Venetoclax.

A deeper dive into mitochondrial biology found that aberrant mitochondrial architecture and bioenergetics were implicated in the apoptotic response to Venetoclax[56]. CRISPR/Cas9 screening further identified a negative association between Venetoclax resistance and mitochondrial chaperonin CLPB, which regulates mitochondrial cristae and metabolism[56]. Chen *et al.*[56] reported overexpression of CLPB in AML which led to tighter mitochondrial cristae lumen. On the other hand, loss of CLPB resulted in wider crista and stimulation of mitochondrial stress response, which in turn triggered cell cycle arrest and lowered the mitochondrial threshold for apoptosis[56]. A combination of CLPB deletion and Venetoclax was found to rescue Venetoclax resistant AML cells even in the presence of TP53 mutation[56]. Another genomic CRISPR-Cas9 knockout screen demonstrated a negative selection of *DAP3*, *MRPL54*, *MRPL17*, *RBFA* genes, which are part of the mitochondrial translation machinery[61]. When mitochondrial protein synthesis was blocked by tedizolid or doxycycline, reduction of AML cell viability was only observed upon co-treatment with Venetoclax, but not with Venetoclax or tedizolid or doxycycline alone[61]. A subsequent investigation by Sharon *et al.*[61] demonstrated that the combination of Venetoclax and tedizolid led to augmentation of the integrated stress response with associated reduction of OXPHOS, and decreased glycolytic capacity, resulting in ATP consumption and cell death.

Monocytic AML in Venetoclax resistance

Several preclinical studies suggested that AML may respond differently to Venetoclax-based therapies, depending on the blast differentiation stage. In the studies discussed below, AML was classified based on cell morphology according to the French, American, and British (FAB) classification system[62]. Guided by flow cytometry, ex vivo drug sensitivity testing on patient samples with primary AML showed a progressive increase in Venetoclax resistance through the cell maturation phase from the most primitive AML (M0) to monocytic AML (M5)[63,64]. Aligned with this observation, analysis of the BEAT AML dataset also noticed

higher Venetoclax AUC in the leukemic blasts with high expression of CD14 and CLEA7A (gene encoding CD369) that are usually present in M4/M5 AML, suggesting Venetoclax resistance in AML with myelomonocytic or monocytic differentiation[37].

Further analysis also indicated different expression levels of alternative BCL-2 anti-apoptotic protein at the selected stages of cell maturation, which correlates with Venetoclax resistance. For instance, a gradual decline in BCL-2 expression with a concurrent increase in MCL-1 expression from M0 to M5 was observed, suggesting a lineage-associate switch to MCL-1 in monocytic AML[63,64]. In addition, the link between high expression of CD14 and CLEA7A, presence of KRAS mutation and increased BCL2A1 were seen in M4/5 AML[37,63,64]. Hence, Venetoclax resistance in AML with myelomonocytic differentiation may additionally be governed by BCL2A1 through upstream mutant KRAS[37,63].

Consistent with these laboratory predictions, analysis of matched patient samples at diagnosis and relapse post-Venetoclax revealed the coexistence of primitive and monocytic features at diagnosis, suggesting developmental heterogenicity within the leukemic blast[64]. Monocytic clone subsequently expanded under the selective pressure of Venetoclax and Azacitidine, with the contemporaneous vanishing of primitive population at the time of relapse[64]. Interestingly, a portion of relapsed monocytic subclone also showed increased HOXA9 expression (similar to that observed in MLL-rearranged leukemia)[65], which was not present in their parental clone[64]. However, both diagnosis and relapsed monocytic clone retained their MCL-1 dependency[64]. These findings suggest the potential role of menin inhibitor or MCL-1 inhibitor to overcome Venetoclax resistance in monocytic disease[64,65].

VENETOCLAX-BASED COMBINATION THERAPIES: LESSONS FROM CLINICAL STUDIES

Venetoclax-based combination therapies

Given low response rates to Venetoclax as a single agent in R/R AML Phase II study, further studies focused on combination regimens. Several lines of clinical trials investigated the combination of Venetoclax with HMA or low-dose cytarabine in both frontline and R/R settings[66-69]. Monotherapy with HMA was commonly used in unfit or older patients with AML, but its benefit is limited by low response rates of ~30% and modest median survival at 8-10 months[70-73]. The synergy between Venetoclax and HMA therapy was reported in preclinical studies. Bogenberger *et al.*[74,75] demonstrated increased sensitivity of HMA after inhibition of BCL-2 family proteins in AML samples. Tsao *et al.*[76] suggested Azacitidine has activity against MCL-1, and it induces synergism with Venetoclax. Subsequent clinical trials validated the clinical efficacy of these regimens.

In a phase 1b study, Venetoclax was combined with either Decitabine or Azacitidine to treat 145 older, newly diagnosed patients unfit for induction chemotherapy. Sixty-seven percent of patients achieved CR/CRi, including 60% of CR/CRi rates in patients with poor cytogenetics risk. The median OS in the study was 17.5 months, and the median duration of response was 11.3 months, with a tolerable safety profile[66]. Following phase 2 study of 10-day decitabine and Venetoclax for intensive chemotherapy ineligible patients, including 70 newly diagnosed AML and 55 R/R AML, confirmed acceptable safety profile and high efficacy with CR/CRi rate of 74%. The study showed a median OS of 18.1 months in newly diagnosed de-novo AML and six months in R/R AML[68]. A randomized phase 3 clinical trial (VIALE-A) comparing Azacitidine monotherapy to combination therapy with Azacitidine and Venetoclax as a frontline treatment confirmed improved response rate and survival in Azacitidine/Venetoclax treated patients compared with Azacitidine alone. CR/CRi rate was 66% in the combination therapy arm compared to 28% in patients who received single Azacitidine[77]. OS was longer in the combination group at 14.7 months with a median event-free survival (EFS) of 9.8 months compared to 9.6 months of OS and seven months of EFS in the single-agent

Azacitidine group.

Cytarabine, a commonly used cytotoxic chemotherapy agent in AML, was likewise shown to have synergism with Venetoclax through inhibition of MCL-1, increased BH3 activity, and upregulation of pro-apoptotic protein, Bim[78]. A phase 1b/2 study of low-dose Cytarabine (LDAC) combined with Venetoclax in newly diagnosed older AML patients demonstrated excellent safety data and a high response rate with CR/CRi of 54% and median OS of 10.1 months[69]. This is encouraging compared to results from prior clinical studies of low-intensity cytotoxic therapy, which showed response rates of less than 20% and median OS of less than six months. Thus, the combination of LDAC and Venetoclax showed significantly improved efficacy compared to conventional chemotherapy[72,79]. In a randomized phase 3 trial in 211 newly diagnosed AML patients ineligible for intensive treatment, the combination of Venetoclax and LDAC was compared to LDAC with placebo. The combination group had a higher CR/CRi rate of 48% compared to 13% of the control group, with an EFS of 4.7 months in the combination group compared to 2 months in the control group[80]. The combination group also had longer OS at 7.2 months compared to 4.1 months in the control group, with a 25% reduction in risk of death[80].

In a phase 1b/2 study of Venetoclax with high-intensity chemotherapy FLAG-IDA (Fludarabine, Cytarabine, granulocyte colony-stimulating factor and Idarubicin), 68 younger AML patients with a median age of 46 years old were enrolled, with 29 de novo and 39 R/R patients[81]. The study demonstrated high clinical efficacy of the combined regimen with median survival not reached at the 12 months median follow-up, and a high minimal residual disease (MRD) negative CR rate of 96% in ND-AML patients and 69% in R/R AML patients. The majority of patients treated with this regimen proceeded towards allogeneic stem-cell transplant (69% of de novo AML and 46% of R/R AML patients)[81].

In another phase 1b study, Venetoclax was given in a dose-escalated fashion up to 600 mg prior to Cytarabine and Idarubicin infusion (5 + 2) in fit older patients with AML age 60 years and above (CAVEAT study)[82]. The combination regimen was well tolerable and efficacious, with ORR of 97% and 43% in de novo AML and secondary AML, respectively. Median OS was 11.2 months, with longer median OS seen in de novo AML of 31.3 months and 29.5 months in patients who achieved CR[82]. This study proved the acceptable tolerability and high efficacy of Venetoclax when given in combination with standard intensive chemotherapy in fit older adults with AML[82].

An ongoing phase 2 trial of the combination of Venetoclax with Cladribine plus LDAC alternating with hypomethylating agents in 48 newly diagnosed older AML patients demonstrated a high CR/CRi rate of 94% with MRD-negativity of 92%. Median OS was not reached over the 11-month median follow-up, and 24% of patients received an allogeneic stem-cell transplant[83].

Molecular landscape associated with response to Venetoclax combination therapies

Analysis of pre-treatment patients' characteristics and paired AML samples pre-treatment and at the time of progression was performed to delineate the molecular patterns associated with treatment response to the combination of Venetoclax with HMA or with low dose Cytarabine (LDAC). In the frontline setting, AML patients with NPM1 or IDH1/2 mutations achieved high rates of CR/CRi and had sustained response for > 12 months[32,66]. Median duration of CR/CRi and median OS was not reached in IDH1/2 or NPM1 mutated patients[66]. Real-world experience supported the observations of IDH1/2 and NPM1 mutations as molecular predictors of response, even in the R/R setting[84,85].

It is noteworthy that deep molecular remissions were seen in patients with NPM1 mutation[32,33]. Highly sensitive RT-qPCR demonstrated MRD clearance in all recruited patients with NPM1 mutation upon treatment with Venetoclax and HMA or LDAC (4/4 cases)[32]. A subsequent study by Tiong *et al.*[33] also reproduced this finding in NPM1 mutated patients with molecular persistence or relapse after treatment with standard intensive chemotherapy. In addition, there was no molecular or hematological relapse observed in NPM1 mutated patients who achieved MRD negative remission after a median follow-up of 11.8 months[33]. Notably, two of the older adult patients unfit for stem cell transplantation (SCT) in this study were alive at 6 and 12 months of follow-up[33], validating Venetoclax-based therapy as a safe and well-tolerated therapy in the frail older adult population. This finding further highlights Venetoclax as a highly efficacious agent to treat NPM1 mutated AML even after intensive chemotherapy failure. A longer follow-up would be needed to evaluate the duration of MRD clearance with Venetoclax based therapy. If indeed durable, a Venetoclax-based regimen could be a well-tolerated and effective option in lieu of SCT in the unfit older population with NPM1 mutated AML. As deep remission was produced by Venetoclax-based therapy after failure of intensive chemotherapy treatment, further studies would be needed to assess Venetoclax-based therapy as a non-inferior or even a superior alternative to intensive chemotherapy in NPM1 mutated younger AML patients prior to transplantation.

Despite the general association with improved response to Venetoclax-based regimens, several points need to be taken into consideration with respect to NPM1 and IDH2 mutations. Despite responding well to combination therapy with Venetoclax and HMA or LDAC, persistent IDH2 mutation is commonly detectable in remission[32]. Although infrequent, new IDH2 mutation has been found at the time of relapse[84], raising questions if adding IDH inhibitor as part of maintenance or consolidative therapy would result in deeper remissions and longer survival. Future studies are needed to address these questions. While NPM1 mutation is generally eliminated by Venetoclax-based therapies, concurrent mutation with FLT3-ITD may confer resistance[32,65]. As NPM1 mutation is associated with heightened HOX/MEIS1 signature[32], the possible role of menin inhibitor merits further investigation and has been shown to enhance anti-AML efficacy of Venetoclax in NPM1-mutant AML models[86]. A combination of menin inhibitor (DS-1594) with Azacitidine and Venetoclax will be evaluated for NPM1c mutated R/R AML in an ongoing phase 1/2 clinical trial (NCT04752163)[65].

In keeping with the response seen with Venetoclax monotherapy, patients with spliceosomal mutation (SRSF2, U2AF1, SF3B1, ZRSR2) obtained a higher response rate to Venetoclax and HMA therapy compared to wild-type patients (CRi/CRh of 28% *vs.* 11%)[87]. Interestingly, the presence of co-mutation seems to influence differential responses seen among genes encoding spliceosome complex. SRSF2 appeared to be enriched with IDH mutations, especially IDH2, compared to the rest of spliceosomal genes cohort[87]. The impressive outcome was seen among SRSF2/IDH1/2 mutated patients with 1-year OS of 100% and 2-year OS of 88%[87]. Patients with SRSF2/IDH1/2 co-mutation also had statistically significant higher survival compared to SRSF2 mutated patients without concurrent IDH mutation, further substantiating the important presence of IDH mutation in driving Venetoclax sensitivity in the SRSF2 group[87]. On the other hand, U2AF1 mutation was enriched with RAS mutation with lower CRc and MRD negative CR rates[87].

Molecular determinants of resistance to Venetoclax combination therapies

In multiple clinical trials of Venetoclax and HMA or LDAC, TP53 mutation was found to be the predominant mutation in the AML patients that did not respond to Venetoclax-based therapy (7/20 cases)[32]. While most of the cases harbored a mutation in the DNA binding domain[88,89], different forms of TP53 abnormalities were observed, including single mutation without TP53 deletion (deletion 17p) and multiple mutations with or without chromosomal abnormality in the TP53 or non-TP53 domain[88]. Complex cytogenetics was seen in the majority of TP53 mutated cases[32,88,89], aligned with a known high

prevalence of TP53 mutation in AML with complex karyotype[90]. These findings underscore TP53 mutation and complex cytogenetics as the most common cause of primary resistance to Venetoclax combination therapy.

Validating preclinical findings[55,59,60], TP53 mutation was associated with significantly lower response rates to Venetoclax and Decitabine combination therapy compared to wild-type group (ORR of 66% *vs.* 89%, CR/CRi of 57% *vs.* 77%, and MRD negative rate of 29% *vs.* 59%)[88]. There was a trend toward improved survival in patients who responded or initially responded prior to relapse compared to primary refractory cases. In responder cases, TP53 mutated AML cohort had a shorter duration of response of 3.5 months *vs.* not reached in the TP53 wild-type AML cohort[88]. These lower responses translated into poor survival with 60-day mortality and median OS of 26% and 5.2 months in the TP53 mutated patients compared to 4% and 19.4 months in the wild-type patients. Interestingly, TP53 mutation burden [variant allele frequency (VAF)] was not proven to be a predictor of response in this mutation cohort, although gain in VAF may be seen at relapse[88]. In line with this finding, single-cell *DNA* sequencing on AML patient samples upon relapse to Venetoclax-based therapy showed expansion of clones containing TP53 mutation under the selective pressure of therapy with a Venetoclax-based regimen[32]. While TP53 mutation may be detected at diagnosis, expansion of new TP53 variants is frequently detected at the time of relapse, with or without concurrent structural loss of 17p locus, suggesting a selection of clones with biallelic TP53 perturbations[32]. Given lower response rates, shorter duration of response, and poor survival, TP53 mutated patients do not derive long-term benefits from Venetoclax-based therapies and should be offered clinical trials whenever feasible. Research to develop novel agents and treatment strategies is urgently needed given the poor prognosis with limited treatment options in this population.

A focused analysis of relapse patterns after Venetoclax-based combination therapy reported the most common emerging mutations in genes involved in signaling pathways (NF1, FLT3-ITD, NRAS, JAK1), in line with the above-mentioned preclinical findings; RNA splicing (U2AF1, U2AF2, SRSF2, ZRSR2), and transcription factors (IKZF1, SETBP1, RUNX1, STAT5A), followed by tumor suppressors (TP53, WT1), and epigenetic modifiers (BCOR, CREBBP)[7]. At relapse, concurrent expansion of clones with different types of activating kinase mutations was shown, including FLT3-ITD, FLT3-TKD, FLT3 N676, RAS, CBL[32]. The emergence of multiple new mutations was also observed in a study by Stahl *et al.*[84], corroborating previous findings. These observations indicate that adaptive resistance may be governed by the complex interaction between various clones rather than single driver mutation. The polyclonal nature of the clonal expansion adds a tremendous challenge to salvage management of AML patients relapsing post Venetoclax-based induction, which is currently associated with extremely poor outcomes and median survival of less than three months[7,32].

Future directions and strategies to mitigate resistance

Current understanding of the mechanisms behind Venetoclax resistance identified in preclinical studies led to the development of several combination strategies that have entered clinical trials, as summarized in Table 1. Amongst these regimens, the combination of Venetoclax with FLT-3 tyrosine kinase inhibitors (TKIs) is under rigorous clinical investigation[91,92]. In a phase 1b trial, the combination of Gilteritinib and Venetoclax reached an ORR of 90% in FLT-3 mutated R/R AML, with similarly high responses in patients who failed prior TKIs[91]. Preliminary result from the "triplet" with Quizartinib, Decitabine and Venetoclax is promising, with a composite response rate (CRc) of 69% and median OS of 7.1 months in the R/R setting, while median OS was not reached in the frontline setting[92]. Recruitment is ongoing for Quizartinib as Venetoclax "doublet" (NCT03735875) or "triplet" (NCT03661307) therapy, and updated results are eagerly anticipated[92,93].

Table 1. Clinical trials evaluating venetoclax-based combination regimens

Drug Regimen	Treatment Category	Mutation (if required for eligibility)	Clinicaltrials.gov identifier	Target Number of patient enrollment[++]	Phase	Year of study initiation
Azacitidine + Venetoclax	Frontline		NCT03466294	42	II	2018
Venetoclax + Decitabine	Both[+]		NCT03404193	400	II	2018
ASTX727 (Decitabine and Cedazuridine) + Venetoclax	Both[+]		NCT04657081	124	I/II	2021
ASTX727 (Decitabine and Cedazuridine) + Venetoclax	Both[+]		NCT04746235	40	II	2021
Venetoclax + Cladribine + LDAC induction followed by Cladribine+ LDAC + Azacitidine	Frontline		NCT03586609	85	II	2018
LDAC + Venetoclax^^	Frontline		NCT02287233	94	I/II	2014
LDAC + Venetoclax *vs* LDAC + placebo	Frontline		NCT03069352	211	III	2017
CPX-351 (Liposome-encapsulated Daunorubicin-Cytarabine) + Venetoclax	Both[+]	In RR subset, (+) RAS pathway activating mutation: KIT, HRAS/NRAS/KRAS, BRAF, CBL or PTPN11 or loss of function mutation of NF1	NCT03629171	52	II	2018
Ivosidenib (IDH1 inhibitor) + Venetoclax +/- Azacitidine	Both[+]	IDH1+	NCT03471260	30	I/II	2018
Enasidenib (IDH2 inhibitor) + Venetoclax	Both[+]	IDH2 (+)	NCT04092179	48	I/II	2020
Gilteritinib (FLT3 inhibitor) + Venetoclax^^	Salvage		NCT03625505	61	I	2018
Gilteritinib (FLT3 inhibitor) + Azacitidine + Venetoclax	Salvage	FLT3	NCT04140487	42	I/II	2019
Gilteritinib (FIT3 inhibitor) + ASTX727 (Decitabine and Cedazuridine) + Venetoclax	Salvage (phase I), Both (phase II)[+]	FLT3	NCT05010122	42	I/II	2021
Quizartinib (FLT3 inhibitor) + Venetoclax	Salvage	FLT3	NCT03735875	32	I/II	2019
Quizartinib (FLT3 inhibitor) + Decitabine + Venetoclax	Both[+]	FLT3	NCT03661307	52	I/II	2018
S64315 (MCL-1 inhibitor) + Venetoclax	Salvage		NCT03672695	40	I	2018
AZD5991 (MCL-1 inhibitor) + Venetoclax**	Salvage		NCT03218683	144	I/II	2017
Pevonedistat (NAE inhibitor) +/- Venetoclax + Azacitidine	Frontline		NCT03862157	40	I/II	2019
Cobimetinib (MEK inhibitor) + Venetoclax; Idasanutlin (MDM2 inhibitor) + Venetoclax^^	Salvage		NCT02670044	88	I	2016

Trametinib (MEK inhibitor) + Azacitidine + Venetoclax	Both[+]	(+) RAS pathway activating mutation in R/R subset	NCT04487106	40	II	2020
Dinaciclib (CDK inhibitor) + Venetoclax	Salvage		NCT03484520	48	I	2018
Alvocidib (CDK inhibitor) + Venetoclax^^	Salvage		NCT03441555	36	I	2018
CYC065 (CDK inhibitor) + Venetoclax	Salvage		NCT04017546	25	I	2019
APR-246 + Venetoclax +/- Azacitidine	Frontline	TP53 +	NCT04214860	51	I	2019
Magrolimab + Venetoclax + Azacitidine or MEC or CC-486 (oral Azacitidine)	Both[+]		NCT04778410	164	II	2021
Magrolimab + Azacitidine + Venetoclax	Frontline (phase I), Salvage (phase II)		NCT04435691	98	I/II	2021
ALX148 (Evorpacept) + Venetoclax + Azacitidine	Both[+]		NCT04755244	97	I/II	2021
TTI-622 (SIRPα-IgG4 Fc) + AZA +/-VEN	Frontline	TP53 +/-	NCT03530683	150	I	2018
Ph I/IIb DS-1594 (Menin inhibitor) +/- Azacitidine + Venetoclax or miniHCVD	Salvage	Presence of MLL rearrangement, NPM1 (+)	NCT03735875	32	I/II	2019

MEC Mitoxantrone, Etoposide, and Cytarabine. *vs.* ^^Completed. **Suspended. [+]Both frontline and salvage therapy. [++]Actual number of patients enrolled for completed studies.

MCL-1 undoubtfully plays an important role in mediating Venetoclax resistance through numerous upstream mediators, both genomic and epigenetic. Hence, targeting MCL-1 directly or indirectly is rational to improve sensitivity to Venetoclax[38-40,46]. The progress in replicating the success of direct MCL-1 inhibitor from bench to bedside has been hindered by observed troponin leak, possibly related to the regulation of mitochondrial homeostasis by MCL-1[47,94,95]. S64315, a direct MCL-1 inhibitor, is being evaluated in the early phase of clinical trial in combination with Venetoclax (NCT03672695)[46,47,93], and additional data on safety profile and efficacy is anticipated.

Several approaches have been pursued to target MCL-1 indirectly, including inhibition of MCL-1 transcription by CKD9 inhibitors, upregulation of NOXA to increase MCL-1 neutralization by NEDD8-activating enzyme (NAE) inhibitor, and targeting RAS-RAF-MEK-ERK (MAPK) pathway to promote MCL-1 degradation by MEK inhibitor and MDM2 inhibitor (a P53 activator)[53,96-98]. Based on synergy seen in the preclinical studies[53,96,98], multiple clinical trials evaluating the combination of Venetoclax and these novel small molecule inhibitors are being conducted, as summarized in Table 1. Several clinical trials have published their preliminary finding. In the newly diagnosed secondary AML population, the combination of Pevonidostat (NAE inhibitor), Azacitidine and Venetoclax (NCT03862157) was safe and efficacious with a CR/CRi rate of 70% and 6-months OS of 82%[99]. A phase 1 clinical trial (NCT02670044) of Venetoclax and Cobimetinib (MEK inhibitor) or Idasanutlin (MDM2 inhibitor) is completed. In R/R/ AML, a preliminary result showed an ORR of 18% in Venetoclax and Cobimetinib cohort[100]. However, Venetoclax 600 mg and Idasanutlin 200 mg arm achieved an ORR of 38%[100]. This finding is encouraging as the achieved response rate was higher compared to Venetoclax monotherapy or combination with LDAC or HMA in R/R population[4,84]. In terms of toxicity, gastrointestinal side effects were predominant with diarrhea, nausea, and vomiting, more with the Cobimetinib combination than idasanutlin[100].

TP53 mutation confers Venetoclax resistance and is associated with worse outcomes[55,84]. MDM2 inhibitor activates wild-type p53 by disrupting p53-MDM2 interaction, which consequently prevents proteosomal p53 degradation, increases p53-regulated, and reduces p53 nuclear export[53,101]. However, MDM2 inhibitor depends on intact p53 function to exert apoptotic activity[102]. In the setting of mutant TP53, APR-246 (eprenetapopt) was developed[103]. Through covalent binding of its reactive electrophile form (methylene quinuclidinone) to mutant p53, APR-246 (methylated PRIMA-1) restores its function and induces p53-dependent apoptosis[103]. A combination of APR-246, Venetoclax and Azacitidine is being evaluated in phase 1 clinical trial (NCT04214860)[93].

Another strategy is harnessing the innate immune system. Leukemic cells evade macrophage-mediated phagocytosis by overexpressing CD47 which binds to the signal regulatory protein alpha (SIRPα) receptor on the macrophage to produce a "do not eat me" signal[3,104]. CD47 becomes a potential therapeutic target as it is highly expressed on LSCs[105]. Several compounds have been developed to block CD47 and SIRPα interaction[104]. Magrolimab (Hu5F9-G4), an anti-CD47 antibody, was the first one to enter the clinical phase[104]. In a phase 1 study, Magrolimab was well tolerated and reduced LSC fraction in patients who achieved a response[106]. Anemia was a notable on-target effect managed by priming and maintenance dose[106]. Importantly, Magrolimab was not observed to augment Azacitidine's toxicity when used in combination[104,106]. This suggests that adding another cytotoxic agent like Venetoclax in combination with Magrolimab is likely safe and synergistic due to the enhanced pro-phagocytic signal[104,106,107]. Clinical trials on the combination of Venetoclax and anti-CD47 antibody (Magrolimab, ALX148), or SIRPαFc fusion protein (TTI-622) are ongoing to validate the safety and efficacy of this novel immunotherapy regimen [Table 1].

Being protected by the bone marrow microenvironment (BMM), eliminating leukemia stem cells (LSCs) is challenging to any cytotoxic regimen, including Venetoclax[108]. In addition to protection, BMM also provides a survival signal and promotes stemness features in resistant AML cells[109,110]. Yu *et al.*[110] recently discovered the role of CD44, an adhesion molecule expressed by LSCs, in governing Venetoclax resistance induced by stimulation of the CXCR4-CXCL12 pathway. Higher MCL-1 expression was also observed after CXCL12 stimulation, which was abrogated by CD44 knockout[110]. Loss of CD44 function also reduced the viability of Venetoclax resistant cells[110]. With several small molecule inhibitors in development[111], a combination of CD44 or CXCR4-CXC12 pathway blockade with Venetoclax-based therapies may be worth pursuing.

CONCLUSIONS

The advent of Venetoclax as a BCL-2 inhibitor has transformed AML management. Multiple Venetoclax-based combination therapies are developed based on current knowledge about mechanisms of responsiveness and resistance to Venetoclax. The heterogenous nature of the disease with rapid and divergent clonal evolution poses a unique challenge when it comes to designing an optimal maintenance or salvage therapy. Molecular profiling would be helpful in personalizing treatment plans, especially in selecting a frontline regimen and prior to changing therapy upon MRD persistence, refractoriness or disease relapse.

Several treatment approaches are being evaluated in clinical trials, with remaining questions to be addressed in the future, all to tackle resistance and improve the durability of response. Current strategies are directed toward maximizing induction therapy by the introduction of "triplets" therapy which incorporates several novel targeted therapies to produce a deeper response upfront with the hope to prevent the emergence of mutation from the surviving clone. Sequential therapy is also of great interest, which can be done

empirically [such as Cladribine + LDAC + Venetoclax alternating with Azacitidine+Venetoclax (NCT03586609)] or adaptively tailored to the molecular signature of the resistance clones. Each approach, however, comes with its own challenge. Triplets therapy may only be suitable for selected genomic subsets and may require Venetoclax's dose adjustment to avoid prolonged myelosuppression. Adaptive sequential therapy is an attractive option. However, this strategy requires sensitive molecular techniques for early detection of rising clone(s), which are not readily available at the moment. Reliance on the limited targetable options further hinders its applicability. Hence, the most feasible option currently is a "shot-gun" combo approach aiming at avoiding resistance through the rotating nature of chemo- or immune-therapy agents with different mechanisms of action, aided by Venetoclax as a universal sensitizer.

Considering these limitations, intensive multi-agent chemotherapy and Venetoclax combination would be the best approach for fit younger AML patients currently as a bridge to stem cell transplantation. Immune-based therapy is another promising approach, given its theoretical efficacy across genomic subsets. Current research is focusing on the benefit of immune-based therapy as "MRD erasers" in maintenance or consolidation therapy in patients with a low disease burden[2,3]. Several agents are currently in development, such as bispecific T-cell engagers (BiTEs), NK engagers, cancer antigen vaccines, and cellular therapies[2,3]. With multiple novel agents added to the AML armamentarium, additional questions remained unanswered, including the efficacy and duration of response of new combination regimens, the molecular pattern of drug response and resistance, duration of therapy after achieving response to treatment, and response to prior regimen after therapy interruption or discontinuation. Future research would be crucial in addressing these questions to evaluate how to optimally use these different types of therapeutic strategies and to identify patients that benefit the most.

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Authors' contributions

Conceptualized, wrote and edited manuscript: Ong F, Kim K, Konopleva M

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Marina Konopleva: Consultant for AbbVie, Genentech, F. Hoffman La-Roche, Stemline Therapeutics, Amgen, Forty-Seven, KisoJi, Janssen; serves as advisory board member for Stemline Therapeutics, F. Hoffman La-Roche, Janssen; holds shares from Reata Pharmaceuticals; honoraria from Forty-Seven and F. Hoffman La-Roche; research funding from AbbVie, Genentech, F. Hoffman La-Roche, Eli Lilly, Cellectis, Calithera, Stemline Therapeutics, Ablynx, Agios, Ascentage, Astra Zeneca, Rafael Pharmaceutical, Sanofi, and Forty-Seven.

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Cancer Drug Resistance

Original Article

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RIP140 regulates *POLK* gene expression and the response to alkylating drugs in colon cancer cells

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Abstract

Aim: The transcription factor RIP140 (receptor interacting protein of 140 kDa) is involved in intestinal tumorigenesis. It plays a role in the control of microsatellite instability (MSI), through the regulation of *MSH2* and *MSH6* gene expression. The aim of this study was to explore its effect on the expression of *POLK*, the gene encoding the specialized translesion synthesis (TLS) DNA polymerase κ known to perform accurate DNA synthesis at microsatellites.

Methods: Different mouse models and engineered human colorectal cancer (CRC) cell lines were used to analyze by RT-qPCR, while Western blotting and luciferase assays were used to elucidate the role of RIP140 on *POLK* gene expression. Published DNA microarray datasets were reanalyzed. The *in vitro* sensitivity of CRC cells to methyl methane sulfonate and cisplatin was determined.

Results: RIP140 positively regulates, at the transcriptional level, the expression of the *POLK* gene, and this effect involves, at least partly, the p53 tumor suppressor. In different cohorts of CRC biopsies (with or without MSI), a strong positive correlation was observed between *RIP140* and *POLK* gene expression. In connection with its effect on POLK levels and the TLS function of this polymerase, the cellular response to methyl methane sulfonate was increased in cells lacking the *Rip140* gene. Finally, the association of RIP140 expression with better overall survival of CRC patients was observed only when the corresponding tumors exhibited low levels of *POLK*, thus strengthening the functional link between the two genes in human CRC.

Conclusion: The regulation of *POLK* gene expression by RIP140 could thus contribute to the maintenance of microsatellite stability, and more generally to the control of genome integrity.

Keywords: Colorectal cancer, genome stability, translesion DNA synthesis polymerase, Pol Kappa, RIP140

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers worldwide and genetic instability exerts a driving role in this malignancy[1]. The mismatch repair (MMR) system is one of the various cellular systems involved in the maintenance of genome integrity through the correction of mistakes that occur during DNA replication. Once impaired (as occurs in 2%-3% of CRC cases with an inherited component including Lynch syndrome[2,3]), it generates microsatellite instability (MSI) and a hypermutated tumor phenotype with a high frequency of point and frameshift mutations[4,5].

In addition to molecular machineries that cope with DNA repair, mammalian cells possess enzymes with translesion DNA synthesis (TLS) activity. The *POLK* gene encodes Polκ, one of the Y-family TLS polymerases, which possesses unique DNA damage bypass capability[6]. Interestingly, despite any associated proofreading exonuclease activity, Polκ was shown to display high accuracy during dinucleotide microsatellite DNA synthesis[7], and *Polκ*-/- mice display a spontaneous mutator phenotype in various tissues[8]. This could be linked to its less open active site compared to other inaccurate specialized DNA polymerases from the Y family[9]. In fact, Polκ possesses a unique structural feature, an extension of its N-terminal region, called N-clasp, that protrudes from the thumb domain and encircles DNA in proximity to a primer terminus[10]. This results in a much more stable complex once Polκ binds to a mismatched DNA substrate, facilitating the extension step across various minor groove distortion or lesion. Besides its role in MSI maintenance together with MMR[7], Polκ seems to prevent DNA damage-induced toxic effects of methylnitrosourea, another process dependent on the MMR system[11]. This link between MMR and Polκ is reinforced by the findings that POLK interacts with MSH2[12] and partially protects human cells from the MMR-dependent cytotoxicity of O6-methylguanine lesions[11].

Our laboratory recently reported that the RIP140 (receptor interacting protein of 140 kDa) gene was involved in the normal and tumoral development of the intestinal epithelium. RIP140, also known as NRIP1 (nuclear receptor-interacting protein 1), was first identified as a transcriptional repressor of nuclear hormone receptors[13,14]. We and others then characterized RIP140 as a coregulator of various transcription factors, including, for instance, E2F[15] or NFKB[16]. The repressive activity of RIP140 involves several inhibitory domains interacting with histone deacetylases[17] and is controlled by different post-translational modifications[18]. Using a mouse model lacking the *Rip140* gene, various physiological processes were shown to be regulated by RIP140, including female fertility[19] and mammary gland morphogenesis[20], fat metabolism[21], pro-inflammatory cytokine response[22], or cognition[23].

In the intestinal epithelium, our laboratory demonstrated that RIP140 inhibits the Wnt/β-catenin signaling pathway and, as a consequence, exerts an anti-proliferative effect[24]. In line with this result, we found that this transcription coregulator inhibits the Paneth cells lineage through the regulation of SOX9 expression and activity[25]. In addition, RIP140 expression decreased in CRC samples compared to the adjacent healthy tissue. In sporadic CRC, RIP140 mRNA and protein levels significantly correlated with better overall survival of patients and were identified as good prognosis markers[24]. More recently, we demonstrated that RIP140 was acting as a transcriptional regulator of *MSH2* and *MSH6* gene expression and was involved in the regulation of the MSI and hypermutator phenotype in CRC cells[26]. Interestingly, a frameshift mutation in the RIP140 coding sequence identified in MSI CRC tumors exhibits a dominant negative activity and correlates with shorter overall survival of patients with advanced CRC.

In the present study, we explored whether RIP140 could regulate POLK as a factor involved in microsatellite stability. We demonstrated that, in both engineered mouse models and human CRC cells, RIP140 positively regulates the *POLK* gene expression at the transcriptional level, at least partly *via* a p53-dependent mechanism. A strong correlation was observed between the expression of the *RIP140* and *POLK* genes in CRC biopsies. Moreover, cells knocked out for the *Rip140* gene were shown to be more sensitive to methyl methane sulfonate (MMS), a drug known to induce DNA lesions activating a POLK response. Finally, our data demonstrate a significant impact of *POLK* gene expression on the prognosis value of RIP140 in human CRC. We propose that the modulation of *POLK* gene expression by RIP140 could reinforce its effect on the maintenance of genome integrity and, more particularly, on the stability of microsatellite sequences.

METHODS

Plasmids

pRL-CMV-renilla and pGL promoters were obtained from Promega (Charbonnieres, France). pEF-cmyc-RIP140 was previously described[27]. pEGFP-RIP140 was a kind gift of Dr. Johanna Zilliacus, (Karolinska Institutet, Huddinge, Sweden)[28]. pEF-cmyc-RIP^{MSI} was generated by mutagenesis using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). pEF-cmyc-RIP^{MSI} was digested with AflII and EcoRV enzymes, and the resulting insert was cloned into pEGFP-RIP140 to create pEGFP-RIP^{MSI}. GFP, GFP-RIP140, and GFP-RIP^{MSI} were PCR amplified and cloned into pTRIPZ previously digested with AgeI and MluI to create pTRIPZ-GFP, pTRIPZ-RIP140 and pTRIPZ-RIP^{MSI}, respectively. All the engineered PCR constructs were sequenced.

Cell culture and transfections

Mouse embryonic fibroblasts (MEFs) derived from wild-type or RIPKO mice previously described[24], and the stably transfected MEFs described by Palassin *et al.*[26] were grown in DMEM-F12 medium supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 100 mg/mL sodium pyruvate, with 40 μg/mL puromycine for selection of stably transfected cells. HCT116, RKO, SW480, and HT29 human colon cancer cells were grown as previously described[26] and stably transfected with the empty pEGFP vector (Clontech®) or with the same vector containing the full-length human RIP140 cDNA. The SW620 human cell line was grown identically. HCT116-GFP and HCT116-RIP140 cells were previously described[24] and grown in McCoy medium and 750 μg/mL G418. Small interfering RNA (siRNA) transfections were performed using INTERFERin® on cells seeded the day before in a 6-well plate (3×10^5 cells per well). Each transfection was performed in triplicate, and interference efficiencies were tested by quantitative RT-PCR.

Animals

C57BL/6J Rip140$^{-/-}$ (RIPKO) mice[19] were given by Pr MG Parker (Imperial College London, London, UK). C57BL/6/129 RIP140 transgenic (RIPTg) mice were generated using the Speedy Mouse® Technology

(Nucleis) by insertion of a single copy of the human RIP140 cDNA at the HPRT locus[24]. All animals were maintained under standard conditions, on a 12 h:12 h light/dark schedule and fed a chow diet ad libitum, according to European Union guidelines for the use of laboratory animals. In vivo experiments were performed in compliance with the French guidelines for experimental animal studies (agreement B34-172-27).

Luciferase assays

HCT116 cells were plated in 96-well plates (2.5×10^4 cells per well) 24 h prior to DNA transfection with Jet-PEI® (275 ng of total DNA). The pGL3-POLK Luc and its truncated mutant pGL3-83 vectors were previously described[29]. The pGL3-29 vector was constructed for this work and co-transfected in HCT116 cells. The pRL-CMV-renilla plasmid (Ozyme®) was used to normalize transfection efficiency. Firefly luciferase values were measured and normalized by the Renilla luciferase activity. Values were expressed as the mean ratio of luciferase activities.

Cell proliferation and cytotoxicity assays

Cells were seeded in quadruplicate at a density of 2×10^3 cells per well. At the indicated time, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich®, St Louis, MO, USA) was added and incubated at 37 °C for 4 h. Formazan crystals were solubilized in DMSO and absorbance read at 560 nm on a spectrophotometer. The results were normalized to the cell density at Day 1. For cytotoxicity assays, cells were seeded in quadruplicate in a 96-well plate (2.5×10^3 cells per well) and exposed the day after to increasing concentrations of cytotoxic drugs orto vehicle alone. The cells were exposed to the drug during six days and cell proliferation was quantified each day using MTT assay. The results were normalized to the mean optical density of the control for each day. MMS and cisplatin were obtained from Sigma-Aldrich®.

Real-time quantitative PCR

Total RNA was extracted from cells using Quick-RNA kit (Zymo Research) according to the manufacturer's instructions. Total RNA (1 µg) was subjected to reverse-transcription using qScript cDNA SuperMix (QuantaBio, VWR). Real-time quantitative PCR (RT-qPCR) were performed with the Roche LightCycler® 480 instrument and the PerfeCTa SYBR Green FastMix (QuantaBio, VWR) and were carried out in a final volume of 10 µL using 0.25 µL of each primer (25 µM), 5 µL of the supplied enzyme mix, 2.5 µL of H_2O, and 2 µL of the template diluted at 1:10 [see Supplementary Table 1 for primer sequences]. After pre-incubation at 95 °C, runs corresponded to 35 cycles of 15 s each at 95 °C, 5 s at 60 °C, and 15 s at 72 °C. Melting curves of the PCR products were analyzed using LightCycler® software to exclude amplification of unspecific products. The results were normalized to different housekeeping gene transcripts (mouse RS9 or human 28S)[30].

Immunoblotting

RIPA solution was used to extract whole-cell proteins. Cell extracts were analyzed after migration of 30 µg protein extract by Western blotting using a primary polyclonal antibody against Polκ (1/1000, Abcam ab57070). Protein quantifications were normalized with the β-actin signal (1/1000, Millipore).

DNA microarray analysis

Published DNA microarray datasets, GSE39582[31] and GSE42284[32], were reanalyzed for RIP140 and POLK expression using the Cancertool database[33]. A transcriptomic dataset from the TCGA-COAD RNA-seq data was also used[4]. Another published DNA microarray study obtained on a cohort encompassing 396 colon tumor samples with MSS and MSI CRC[34] was also reanalyzed for RIP140 and POLK mRNA expression. Statistical significance was assessed using a Spearman correlation analysis. Correlation between

RIP140 and *POLK* gene expression was also studied using another cohort[35]. RNA sequencing data from the TCGA[4] were reanalyzed using Cox proportional hazard regression[36]. RNAseq data obtained from CRC samples from the TCGA dataset were analyzed as described previously[37]. The Kaplan-Meier method was used to estimate overall survival (OS) calculated from the diagnosis until death. Patients lost to follow-up were censored at the time of last contact.

Statistical analysis

All experiments were conducted independently at least three times. The results were expressed as the mean ± standard deviation (S.D.). Statistical comparisons were performed with Mann-Whitney or Spearman tests. For the Cancertool database analysis, a Pearson's test was performed for the correlation analyses. A probability level (*P*-value) of 0.05 was chosen for statistical significance.

RESULTS

RIP140 regulates *POLK* gene expression in mouse models

To explore the role of RIP140 on the regulation of the *POLK* polymerase gene expression, we first used transgenic mice in which the *Rip140* gene was either knocked out (RIPKO mice) or overexpressed (RIPTg mice) [24]. As shown in Figure 1A, a significant decrease in the levels of *Pol*κ mRNA was observed in the intestinal epithelium of RIPKO mice, whereas an increase was noted in RIPTg mice as compared to wild-type animals (WT). These regulations appeared specific since the expression of other TLS polymerase genes from the Y subfamily such as *PolI* did not vary between the different genotypes [Figure 1A and data not shown]. The steady-state levels of Polκ mRNA were also significantly reduced in immortalized MEFs derived from the RIPKO mice as compared to cells isolated from WT animals [Figure 1B]. Altogether, these results demonstrate that RIP140 positively controls the expression of the Polκ gene in mouse cells and tissues.

RIP140 regulates *POLK* gene expression in CRC cells

To check whether this regulation can be observed in human cells, we then analyzed the effect of RIP140 on *POLK* gene expression in human CRC cell lines. We confirmed the increased expression of the *POLK* gene at the mRNA levels in HCT116 cells either stably overexpressing RIP140 [Figure 1C] or transiently transfected with a RIP140 expression vector [Figure 1D]. We also unveiled that silencing the expression of the *RIP140* gene in HCT116, RKO or HCT29 CRC cells significantly affected *POLK* mRNA abundance [Figure 1E and F and Supplementary Figure 1] as well as the Polκ protein levels in RKO cells [Figure 1G].

Transcriptional regulation of *POLK* gene transcription in CRC cells

To decipher the mechanisms underlying the positive regulation of *POLK* gene expression by RIP140, we set up transient transfection experiments of HCT116 cells using the POLK Luc reporter construct encompassing the proximal promoter region of the *POLK* gene [Figure 2A]. As observed in Figure 2B, RIP140 significantly increased, in a dose-dependent manner, the luciferase activity driven by the *POLK* gene promoter, thus supporting a positive transcriptional regulation by RIP140. The same effects were observed in other CRC cells including RKO, SW480, and SW620 cells [Figure 2C].

Mechanism of the transcriptional regulation by RIP140

Although RIP140 was first identified as a transcriptional repressor, we and others reported positive regulation of gene expression (for a review, see Ref.[38]). In particular, we described a transcriptional activation of gene expression by RIP140 through Sp1-mediated mechanisms[39]. Since the *POLK* gene promoter exhibits Sp1 binding sites, we tested if the deletion of these sites affected the transcriptional response to RIP140 ectopic expression. As shown in Figure 2D, the induction of luciferase activity by RIP140 was significantly reduced when we used a short reporter construct (*POLK* 29 Luc), suggesting that

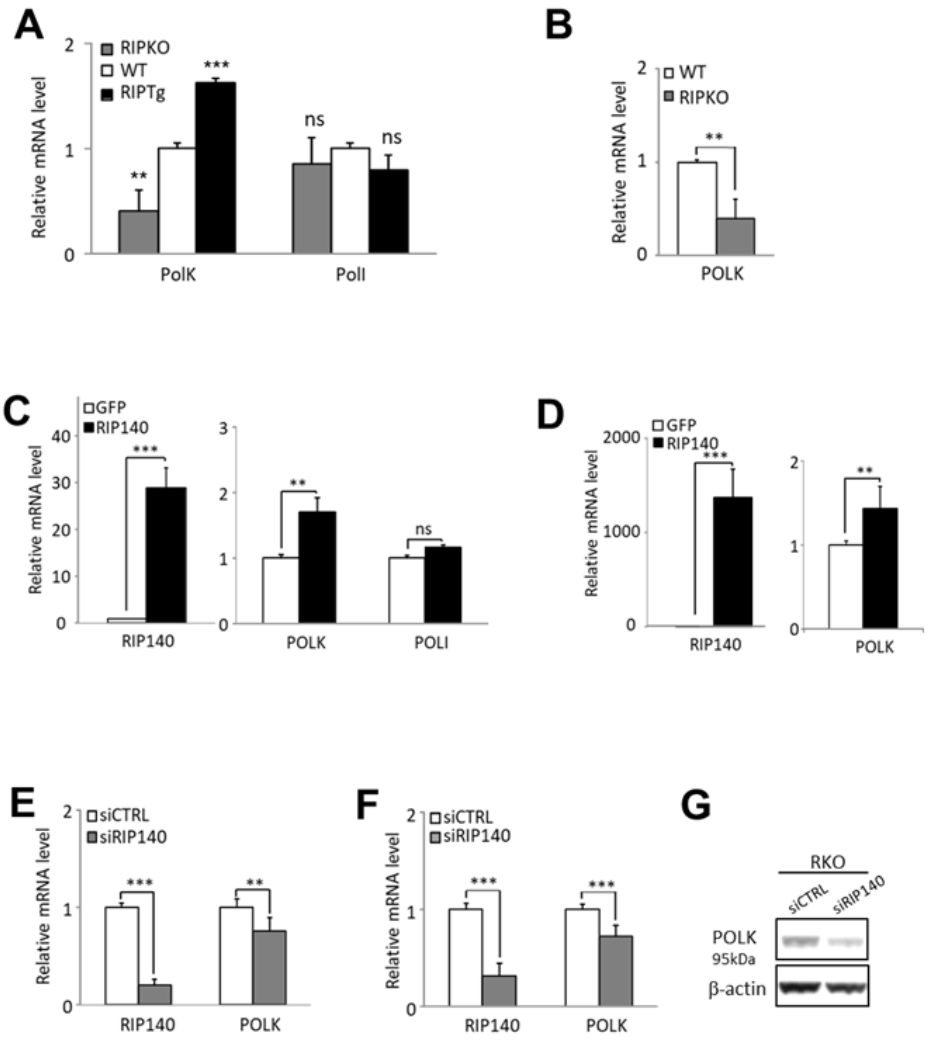


Figure 1. RIP140 regulates *POLK* gene expression. (A) RT-qPCR analysis of Polκ and PolI mRNA in the intestinal epithelium of mice lacking the Rip140 gene (RIPKO) and in transgenic mice overexpressing RIP140 (RIPTg) as compared to their WT littermates. The results for each gene are given in arbitrary units (AU) and expressed in fold change ± S.D. relative to WT after normalization to mouse RS9 mRNA. (B) The same as in (A) in immortalized MEFs from WT or RIPKO mice. (C) HCT116 cells were stably transfected with the pEGFP-RIP140-expressing vector (HCT-RIP140) or pEGFP alone (HCT-GFP). RIP140, POLK, and POLI mRNA levels were quantified by RT-qPCR. The results are expressed relative to GFP cells after normalization to human 28S mRNA. (D) The same as in (C) in HCT116 cells transiently transfected with a RIP140 expression vector. (E) RIP140 and POLK mRNA levels were quantified by RT-qPCR in HCT116 cells transiently transfected with siCTRL or siRIP140 siRNAs as indicated. The results are expressed as fold change ± S.D. relative to siCTRL after normalization to 28S mRNA ($n = 3$ independent experiments for each condition). (F) The same as in (E) performed in RKO CRC cells. (G) *POLK* expression analysis by Western blot of whole cell extract from RKO cells 48 h after transient siRNA transfection. Quantifications are expressed in AU after normalization to β-actin, used as a control of protein migration. A Mann-Whitney test was used for statistical analysis (ns = not significant, $^{**}P < 0.01$, $^{***}P < 0.001$).

the regulation of *POLK* gene expression by RIP140 might be, at least in part, mediated by Sp1.

Interestingly, a close correlation between elevated POLK expression and p53 inactivation was previously reported in lung cancer tissues[40]. This effect is likely due to the direct role of p53 on the *POLK* gene since p53 strongly inhibits the *POLK* promoter activity in lung cancer cells, while this activity is much higher in $p53^{-/-}$ MEF than in $p53^{+/-}$ and $p53^{+/+}$ MEFs[40]. Therefore, we checked whether the positive regulation of *POLK* gene expression by RIP140 was dependent on p53. By using HCT116 cells expressing or not the *TP53* gene,

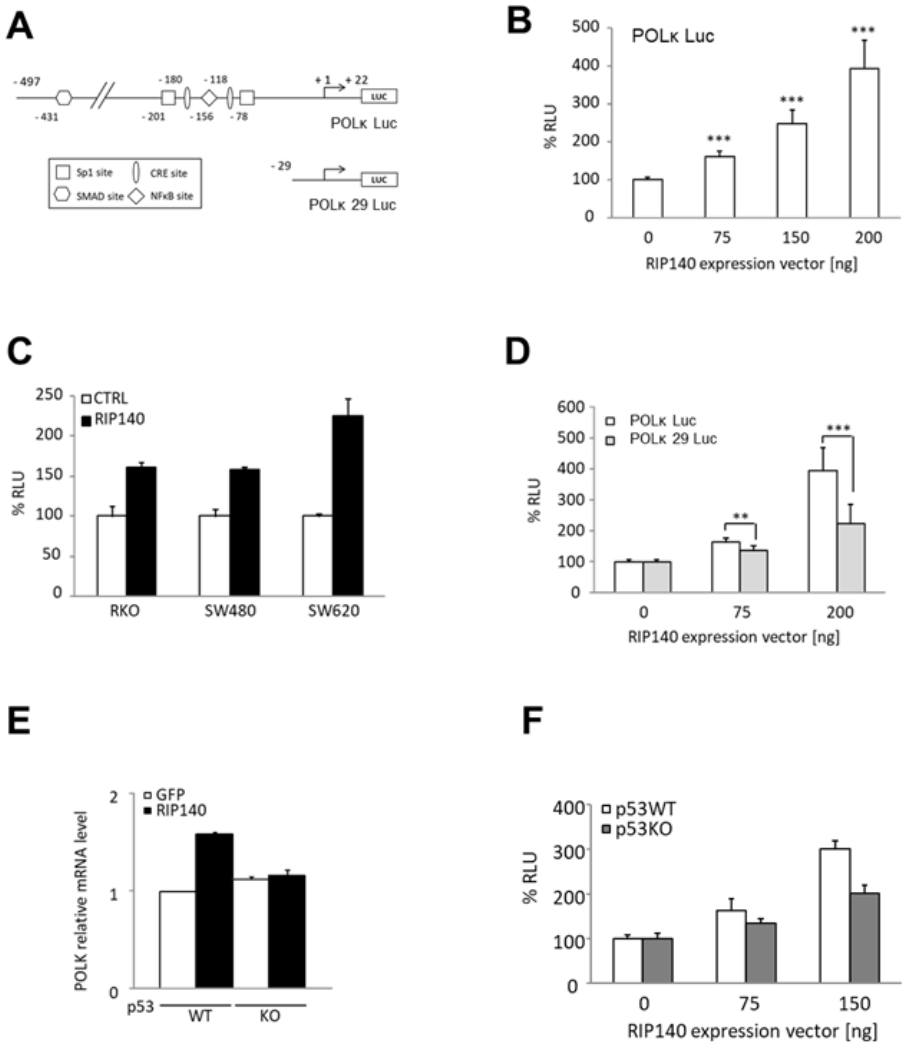
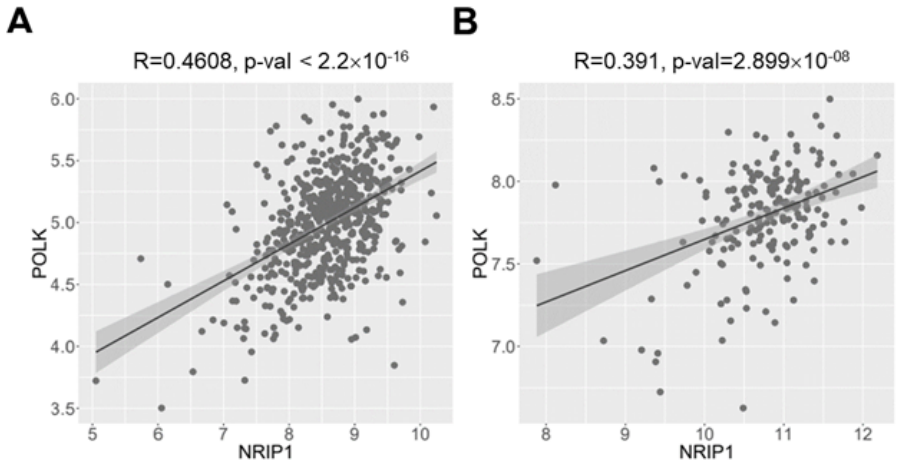


Figure 2. RIP140 regulates POLK at the transcriptional level in CRC cells. (A) Schematic representation of *POLK* proximal promoter cloned in a pGL3 promoter and the putative regulatory sites identified as well as the *POLK* 29 Luc deletion mutant. (B) The *POLK* Luc construct was transiently co-transfected into HCT116 cells with increasing doses of a pEF-cmyc-RIP140 expression vector and pRL-CMV-renilla as an internal control. The reporter activity is presented as relative luciferase activity (RLU) as mean ± S.D. (n = 3 independent experiments). (C) The same reporter assay as in (B) performed in RKO, SW480, and SW620 cells. (D) Luciferase reporter assay performed with the two reporter vectors described in (A), in HCT116 cells. Values, expressed as a percent of control, are means ± S.D. (n = 3 independent experiments). (E) The effect of RIP140 ectopic expression on POLK mRNA levels after transient transfection in p53WT or p53KO HCT116 cells. (F) Transactivation of the *POLK* gene promoter by increasing doses of a RIP140 expression vector in p53WT or p53KO HCT116 cells. A Mann-Whitney test was used for statistical analysis (**P < 0.01 and ***P < 0.001).

we found that, indeed, the regulation of the *POLK* gene by RIP140 was abolished in HCT116 $p53^{-/-}$ cells when monitored at the mRNA level [Figure 2E] or using a luciferase reporter assay [Figure 2F], supporting that p53 is involved in the RIP140-mediated regulation of *POLK* levels.

Correlation of gene expression in human CRC biopsies

To validate in human CRC biopsies the expression data obtained in mouse tissues and in CRC cells *in vitro*, we used the Cancertool database[33] to reanalyze the published Affymetrix DNA microarray data from two cohorts, namely GSE39582 containing 585 tumors[31] and GSE42284 with 188 samples[32]. As shown in Figure 3A and B, and in perfect agreement with the data from mice, the results clearly show a significant positive correlation of *RIP140* mRNA levels with those of *POLK* in CRC biopsies. We also reanalyzed



C

	POLK	
	r	p
All samples (n=415)	0,65	<2.2×10^{-16}
C1 group (n=46)	0,79	<9×10^{-11}
C2 group (n=75)	0,49	<7×10^{-6}
C3 group (n=66)	0,71	<2×10^{-11}
C4 group (n=51)	0,80	1,00×10^{-12}
C5 group (n=100)	0,47	<8×10^{-7}
C6 group (n=77)	0,72	<2×10^{-13}

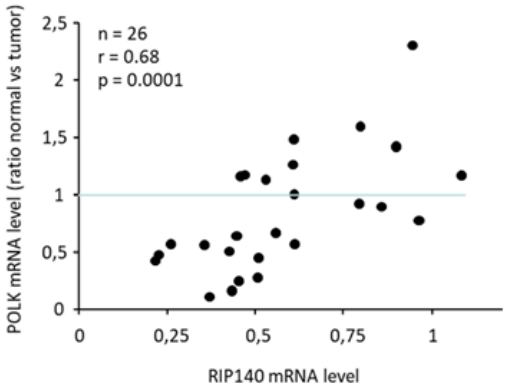


Figure 3. Correlation of *RIP140* and *POLK* gene expression in human CRC samples. (A) Analysis of *RIP140* and *POLK* correlation using the mRNAs expression from the Cancertool database in the GSE39582 cohort[31]. The correlation coefficient and the p-value (Pearson's test) are indicated. (B) Same as in (A) with the cohort GSE42284[32]. (C) Correlations between *RIP140* and *POLK* gene expression performed with TCGA RNA-Seq cohort (n = 415)[4] showing the correlations found in the different CRC molecular subtypes described in this cohort. (D) Correlation between *POLK* and *RIP140* gene expression in a cohort comparing the expression of "DNA replication" genes in CRC and in the adjacent normal mucosa (n = 26)[35].

another transcriptomic dataset from the TCGA-COAD RNA-seq data[4] obtained on 415 samples, which confirmed a significant correlation between RIP140 and POLK mRNA levels (r = 0.65; $P < 2.2 \times 10^{-16}$) [Figure 3C]. This dataset was further reanalyzed, taking into account the different molecular subtypes[31]. This allowed us to show a significant correlation of *RIP140* gene expression with that of *POLK* in the six different subgroups, in particular in the C2 group which corresponds to the MMR deficient subgroup. Finally, we confirmed these data in another cohort comparing the expression of "DNA replication" genes in CRC and in the adjacent normal mucosa[35]. We confirmed that *RIP140* gene expression strongly decreased in the tumor (data not shown) and that there was a strong correlation in the expression ratio (normal *vs.* tumoral) of the two genes (r = 0.68; P = 0.0001) [Figure 3D].

Effect of the RIP^{MSI} mutation on the regulation of *POLK* gene expression

We recently demonstrated that RIP140 plays an important role in controlling microsatellite instability through the regulation of *MSH2* and *MSH6* genes[26]. To compare the correlation between *RIP140* and *POLK* gene expression in microsatellite stable (MSS) and instable (MSI) CRC tumors, we reanalyzed a transcriptomic dataset from 396 human CRC with both types of tumors[34]. As shown in Figure 4A, we observed a very significant positive correlation between *RIP140* and *POLK* mRNA levels in the whole cohort ($r = 0.74$; $P = 5.2 \times 10^{-69}$), thus confirming the results shown in Figure 3. Interestingly, *POLK* and *RIP140* gene expression were significantly correlated in both MSS and MSI tumors [Figure 4B].

In MSI CRC, we identified a frameshift mutation in the RIP140 coding sequence[26]. This mutation (called RIP^{MSI}) generated a truncated protein which impaired the biological activity of the RIP140 protein. As shown in Figure 4C, the RIP^{MSI} protein was found to be less efficient than the WT protein to increase *POLK* gene expression. The same results were obtained on endogenous gene expression in HT29 CRC cells overexpressing wild-type or mutated RIP140 [Figure 4D]. Interestingly, the RIP^{MSI} mutant exhibited a dominant negative effect since its ectopic expression significantly decreased *POLK* mRNA accumulation only in WT MEFs, which express normal levels of RIP140, and not in RIPKO MEFs, which no longer express the *Rip140* gene [Figure 4E].

Functional consequences of the regulation of POLK by RIP140

Since the *POLK* gene is involved in the cellular response to cytotoxic drugs including MMS and cisplatin through a TLS replication process[41], we next measured the importance of RIP140 on MMS and cisplatin sensitivity by comparing IC_{50} ratios between RIPKO cells and their WT counterparts. As shown in Figure 5A and B, we observed a significant increase of sensitivity to MMS and cisplatin when the *RIP140* gene was knocked out (IC_{50} ratio WT/RIPKO = 9.7, $P < 0.05$ for MMS and IC_{50} ratio WT/RIPKO = 1.9, $P < 0.001$ for cisplatin). Altogether, these data suggest that the regulation of *POLK* gene expression by RIP140 can affect the cellular responses to various alkylating agents, including anticancer drugs.

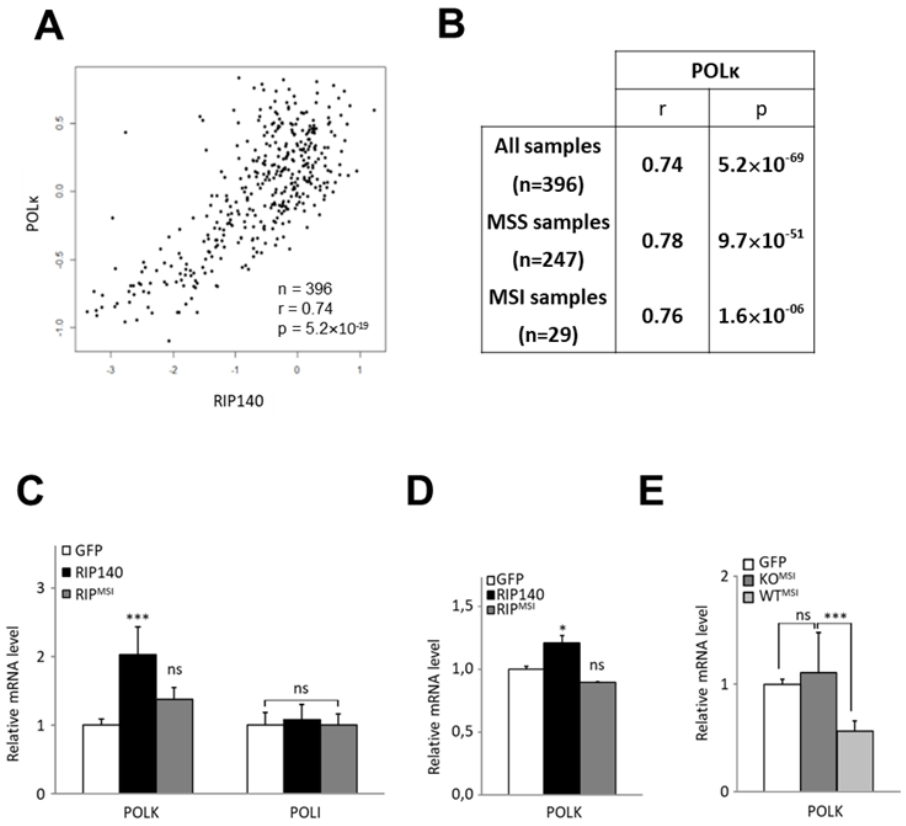
POLK expression impacts the prognosis value of RIP140 in CRC tumors

Using the Kaplan-Meier Plotter database, we then investigated whether *POLK* levels contribute to the good prognosis value of RIP140 that we previously reported in CRC patients[24]. We reanalyzed RNAseq data obtained from 452 patients with colon adenocarcinoma that we separated into two groups of 226 patients, each with low and high *POLK* gene expression in the corresponding tumors using the median as a cutoff value. As shown in Figure 6A and B, a statistically significant association of high expression of RIP140 with a decreased risk of death in CRC patients was observed when their tumor exhibited low *POLK* gene expression [Figure 6A] but not in tumors with high *POLK* gene expression [Figure 6B]. As a control, no differences were observed when the 452-patient cohort was stratified based on the median value of *POLI* expression [Figure 6C and D]. Collectively, all the data obtained in this work strongly support a molecular and functional link between RIP140 and POLK in CRC.

DISCUSSION

Colorectal cancer is a frequent neoplasm with high genomic instability including MSI due to defects in the MMR system. We previously described that the transcription factor RIP140 was a key factor in the regulation of intestinal homeostasis and tumorigenesis[24]. We also showed that RIP140 could be involved in microsatellite stability through the regulation of *MSH2/MSH6* gene expression[26].

In the present study, we demonstrated that RIP140 also strongly regulates *POLK* gene expression, in both mouse and human cells and tissues. Data obtained using luciferase reporter assays demonstrate that this regulation took place at the transcriptional level and implicated the proximal region of the *POLK* gene.



	POLκ	
	r	p
All samples (n=396)	0.74	5.2×10^{-69}
MSS samples (n=247)	0.78	9.7×10^{-51}
MSI samples (n=29)	0.76	1.6×10^{-06}

Figure 4. RIP140 and POLK in MSI CRC tumors. (A) Correlation between *RIP140* and *POLK* gene expression in 396 colorectal adenocarcinomas[34]. (B) Statistical significance was assessed using a Spearman correlation analysis on this cohort, containing 247 microsatellite stable (MSS) and 29 microsatellite instable (MSI) samples. Spearman correlation coefficients between *RIP140* and *POLK* gene expression are indicated for the whole cohort, as well as MSS and MSI samples. (C) mRNA quantification of the *POLK* and *POLI* genes in MEF RIPKO stable cells expressing either the GFP (white box) or the GFP fused wild-type form (black box) or the RIP^{MSI} mutant form (grey box) of RIP140. (D) mRNA quantification of the *POLK* gene in HT29 CRC cells transiently transfected with pEGFP, pEGFP-RIP140, or pEGFP-RIP^{MSI} expression vectors. (E) Analyses of the mRNA expression of the *POLK* gene in MEFs cells stably transfected with the human expression vector of RIP^{MSI} in a RIP140 wild-type background (MEF WT) or knock-out (MEF RIPKO) as compared to the control transfected with a GFP expressing vector in each condition. A Mann-Whitney test was used for statistical analysis (ns = not significant, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

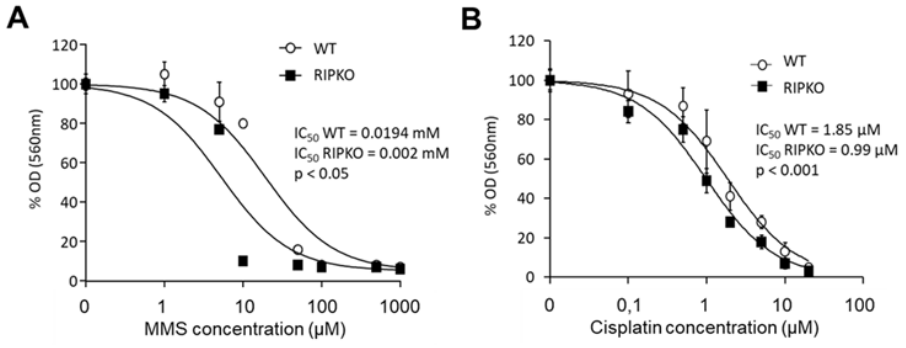


Figure 5. *RIP140* expression affects the response to cytotoxic drugs. (A) MEF WT and RIPKO cells were exposed or not to increasing doses of methyl methane sulfonate (from 0.5 µM to 1 mM). The optical density of diluted formazan crystals was expressed in percentage relative to the control. IC_{50} values of each cell type are mentioned together with the p-value of the nonlinear regression performed with GraphPad® software, allowing the comparison of IC50 between each cell type dose-response (IC_{50} ratio WT/RIPKO = 9.7, $P < 0.05$). (B) The same as in (A) with increasing doses of cisplatin (from 0.1 to 20 µM) (IC_{50} ratio WT/RIPKO = 1.9, $P < 0.001$). Mouse embryonic fibroblasts wild-type.

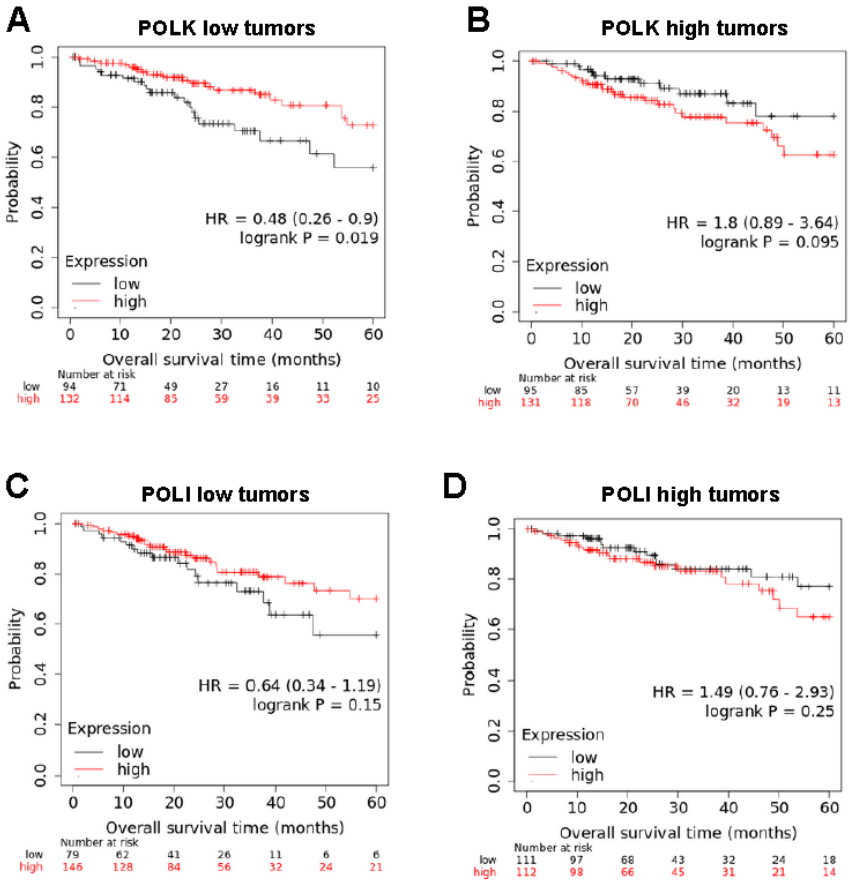


Figure 6. The prognosis value of RIP140 in CRC tumors is dependent on *POLK* gene expression. (A) Kaplan-Meier analysis of the cumulative OS of patients with low *POLK* gene expression was performed into the groups exhibiting low or high *RIP140* gene expression. A log-rank test was used for statistical analysis. (B) The same analysis as in (A) with tumors expressing high *POLK* gene expression. (C,D) A similar study was performed using *POLI* levels to define the two subcohorts.

Several transcription factors including p53[40], Sp1, and CREB[29] or AhR[42] have been shown to control the expression of the *POLK* gene, and some of them have their activity regulated by RIP140[43]. The data presented herein suggest that p53 is a good candidate to mediate, at least partly, the regulation of *POLK* gene expression since the positive effect of RIP140 is lost in HCT116 cells no longer expressing the *TP53* gene. Moreover, it has been shown that the HDAC inhibitor Trichostatin A was able to induce *POLK* gene expression[29]. Since RIP140 strongly interacts with HDACs[44], it is possible that HDAC sequestration out of the *POLK* gene promoter also participates in the positive effect exerted by RIP140 on *POLK* gene transcription as already demonstrated for other transcription factors[39]. Further work will be necessary to fully decipher the mechanisms used by RIP140 to transcriptionally control *POLK* gene expression.

Cell survival relies on a subtle equilibrium between accurate genomic DNA replication and less stringent DNA synthesis often linked to DNA damage. POLK plays an important and critical role in such equilibrium by contributing to several important DNA transaction pathways. These include translesion synthesis, which allows stalled replication forks to bypass a lesion and restart[45]; the replication checkpoint[46], a crucial pathway that regulates the replication stress response to a large array of insults that cause replication arrest; and the synthesis of intrinsic non-B microsatellite DNA[7]. Consistent with these multiple and complex roles, both overexpression of *POLK* (associated with advanced disease stages and shorter survival in patients with glioma[47] and non-small cell lung carcinomas[48]) and under-expression of *POLK* (frequently observed

in colorectal, lung, stomach, and breast cancers[29,35,49,50]) have been documented to lead to genetic instability. Indeed, excessive Polκ can interfere with fork progression and trigger a mutator phenotype and chromosome instability, while downregulation of Polκ expression can affect fork progression and induce replicative stress. Since both situations can confer a selective growth advantage during cancer cell evolution, this underlines the importance of tight regulation of *POLK* gene expression at the transcriptional level for the maintenance of genome integrity. Therefore, our discovery here establishing the role of RIP140 in *POLK* gene regulation not only may explain why POLK is abnormally expressed in cancer, notably in CRC, but can also clarify why cancers cells respond differentially to anticancer genotoxic drugs that target DNA replication forks. This last point is illustrated in the present work by cell sensitivity to MMS treatment, which can be modulated by RIP140.

In addition, it is worth mentioning that the regulation of *POLK* gene expression could intensify the phenotypic consequences of the regulation of *MutSα* gene expression by RIP140[26]. Indeed, this TLS polymerase displays a high accuracy during dinucleotide microsatellite DNA synthesis and has been proposed to play a role in the maintenance of microsatellite stability[7]. Moreover, Polκ interacts with MSH2[12] and could therefore participate in the control of MutSα activity. Defining the precise involvement of *POLK* gene induction in the effect of RIP140 on the maintenance of genome integrity in colon cancer cells (in particular on the stability of microsatellites) and on their resistance to anticancer drugs will obviously require further work. It would also be interesting to decipher whether, as it is suggested for the MSH2 protein[51], POLK could be involved in the RIP140-mediated transcriptional regulation of gene expression.

Altogether, the present data suggest that *POLK*-defective cells exhibit an altered DNA replication program, thus explaining the spontaneous genetic alterations observed in *POLK*-deficient mice[8]. Transcriptional deregulation of *POLK* gene expression may therefore participate in intestinal tumorigenesis and account, at least in part, for the tumor suppressor role of RIP140 that we previously suggested.

DECLARATIONS

Authors' contributions

Substantial contributions to conception and design of the study: Cavaillès V, Castet-Nicolas A
Data acquisition, as well as technical and material support: Palassin P, Bonnet S, Lapierre M, Jalaguier S, Teyssier C, Pillaire MJ, Hoffmann JS
Data analysis, statistical analysis and interpretation: Palassin P, Lapierre M, Cavaillès V, Castet-Nicolas A
Bioinformatic analysis: Győrffy B
Manuscript writing: Palassin P, Lapierre M, Hoffmann JS, Cavaillès V, Castet-Nicolas A
The manuscript draft was inserted in the P. Palassin PhD thesis, and not published elsewhere
Manuscript reviewing and editing: Pillaire MJ, Jalaguier S, Teyssier C
Study supervision: Cavaillès V, Castet-Nicolas A

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

In vivo experiments were performed in compliance with the French guidelines for experimental animal studies (agreement B34-172-27 for IRCM and CEEA-LR-12158 for the experiments, obtained from the ethics committee for animal experimentation).

Consent for publication

Not applicable.

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Cancer Drug Resistance

Perspective

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Ovarian cancer recurrence: is the definition of platinum sensitivity modified by PARPi, bevacizumab or other intervening treatments? : a clinical perspective

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Abstract

In view of the high risk of recurrent disease in stage III and IV ovarian cancer following primary first-line chemotherapy, a variety of maintenance and consolidation treatment strategies have been developed. These have included: radiation, intravenous or intraperitoneal chemotherapy, targeted therapies, and immunotherapy. Popular at this time is the use of Poly-adenosine ribose polymerase (PARP) inhibitors and bevacizumab as maintenance therapy. What effect these maintenance or consolidation therapies have on subsequent response to therapy, specifically platinum-based chemotherapy, is only beginning to be studied. In this manuscript, we review the impact of PARP inhibitors and bevacizumab as well as radiation and maintenance chemotherapy on subsequent response to treatment. Prior use of bevacizumab does not appear to adversely affect subsequent response to platinum-based chemotherapy or platinum-based chemotherapy with bevacizumab. Prior therapy with PARP inhibitors induces platinum resistance to subsequent platinum-based therapy and negates classic predictors of response such as platinum-free interval and breast cancer susceptibility gene (BRCA) mutational status.

Keywords: Maintenance, consolidation, PARP inhibitors, bevacizumab, radiation, chemotherapy

INTRODUCTION

Because of a lack of early detection methodology, most patients with ovarian cancer present with advanced-stage (stage III and IV) disease. Despite high complete response rates to surgery and primary chemotherapy, recurrence occurs in approximately 80% of these patients. Because of this very high recurrence rate, a variety of maintenance/consolidation strategies following completion of first-line chemotherapy have been tested in this patient population. Maintenance/consolidation strategies have included radiation, intravenous or intraperitoneal chemotherapy, targeted therapies, and immunotherapy[1,2].

Despite maintenance/consolidation strategies, recurrence remains high. Little is known about the interaction of maintenance/consolidation therapies and their impact on subsequent response to treatment. In the era before maintenance therapy, once a patient recurs, a major factor in choosing a second-line therapy is the patient's potential for retreatment with a platinum compound. In 1992, Markman and Hoskins were the first to recognize the importance of defining the recurrent ovarian cancer population and proposed the definition of platinum-sensitive and platinum-resistant disease[3]. Patients that progressed on therapy had less than a partial response or recurred within 6 months were considered platinum-resistant, while patients who had a previous partial or complete response and did not require chemotherapy for 6 months were considered potentially platinum-sensitive. Subsequent definitions have subdivided patients who recurred within 6 months to those who recur between 0 and 3 months as platinum-refractory *vs.* 3 to 6 months as platinum-resistant[4]. Additionally, platinum-sensitive patients have been subdivided into those who recur in 6 to 12 months as partially platinum-sensitive and those who recur more than 12 months as fully platinum sensitive. Other authors feel platinum sensitivity is a continuum without a strict time cut-off[5]. The duration of time off of platinum defines the platinum-free interval.

Platinum-containing drugs act mainly to induce DNA damage which activates a DNA damage response[6]. Tumors with deficiencies in homologous recombinant DNA repair are unable to repair platinum-induced DNA damage and undergo apoptosis. Approximately 20% of patients with ovarian cancer have a hereditary germline mutation in the Fanconi Anemia-BRCA pathway, which increases the risk of ovarian cancer and increases their response to chemotherapy[7]. An additional 30% of the patients with the most common type of ovarian cancer (high-grade serous carcinoma) may also have somatic mutations in the tumor, which increases the likelihood of responding to chemotherapy and are classified as homologous recombinant deficient (HRD)[8]. In addition to the platinum-free interval, the BRCA mutational and HRD status also is associated with improved response to second-line chemotherapy. Among patients with ovarian cancer patients with BRCA mutations, Tan *et al.*[9] reported higher response rates (CR/PR) after second-line (70.6 *vs.* 38.7%; $P = 0.035$) and third-line (64.3 *vs.* 8.7%; $P = 0.001$) platinum-based chemotherapy compared to non-BRCA mutated patients.

Poly-adenosine ribose polymerase (PARP) enzymes play critical roles in the repair of single-strand DNA breaks (SSB) and maintenance of genomic integrity of a cell[10]. Like platinum compounds, inhibitors of poly-adenosine ribose polymerase (PARPis) have their greatest oncologic effect on tumors with germline BRCA mutations and HRD. However, even in *BRCA* mutated patients, response to PARP is closely related to the patient's platinum sensitivity. Fong *et al.*[11] demonstrated a variable clinical benefit rate across the platinum-sensitive, resistant, and refractory subgroups (69%, 45%, and 23%, respectively). This suggests a common mechanism of drug resistance. Ang *et al.*[12] made the first report of the clinical outcome of post PARP inhibitor chemotherapy. Among 48 BRCA mutated patients who subsequently received post olaparib platinum-based chemotherapy, a response rate of 40% was noted. The authors suggested non-overlapping mechanisms of drug resistance. More recently, a number of authors have reported decreased response to second- and third-line platinum-based chemotherapy in BRCA mutated patients following PARPi therapy

Table 1. Cecere *et al.* [13] studied 234 BRCA mutated patients that received olaparib after at least two platinum-based regimens. Eighty-eight percent of these patients received 2-4 platinum-based regimens. Sixty-six of these patients received post olaparib chemotherapy. These patients were stratified by their platinum-free interval. Patients with platinum-free intervals of greater than 12 months predominantly (78%) received a platinum regimen. Fifty-two percent of patients with platinum-free intervals of 6-12 months received a platinum regimen. No patients with platinum-free intervals of < 6 months received a platinum regimen. The response rates were low for all platinum-free intervals. Baert *et al.*[14] evaluated the rate of disease progression to third-line platinum chemotherapy among 35 BRCA mutated and non-mutated patients. Prior PARP inhibitor treatment significantly increased the frequency of disease progression by 40% *vs.* 9% in non-PARP inhibitor-treated controls. Frenel *et al.*[15] evaluated the progression-free survival following third-line platinum-based chemotherapy among BRCA mutated patients enrolled in SOLO-2. The progression-free survival to third-line platinum-based chemotherapy was significantly reduced in the olaparib-treated patients by 7 *vs.* 14.3 months in the placebo-treated controls. Rose *et al.*[16] studied BRCA mutated patients who received second- or third-line platinum-based chemotherapy. Patients who received a PARP inhibitor following first- or second-line chemotherapy had a poorer progression-free survival to second- or third-line platinum-based chemotherapy, respectively (8 months *vs.* 19.1 months). This study also demonstrated that there was no prognostic significance to the platinum-free interval as the progression-free survival among BRCA mutated patients with prior platinum-free intervals of 6 to 12 months, 12 to 24 months, or more than 24 months was the same. Additionally, there was no prognostic significance to BRCA mutational status as the progression-free survival of patients to subsequent platinum-based chemotherapy who were BRCA mutated or non-BRCA mutated following prior PARP inhibitor exposure was the same.

These findings may explain why PARP inhibitors demonstrate significant improvements in progression-free survival but not overall survival. While the mechanisms of platinum and PARP inhibitor resistance have only begun to be determined, Johnson *et al.*[17] did demonstrate stabilization of mutant *BRCA*1 protein confer PARP inhibitor and platinum resistance.

Another unresolved issue regarding PARP inhibitor utilization is when PARP inhibition therapy should be instituted. In study 19, which studied olaparib maintenance after second-line platinum-based chemotherapy, a significant improvement in overall survival was only evident when placebo-treated patients who received subsequent PARP inhibitor therapy were excluded from the analysis[18]. This implies that delayed PARP inhibition may be just as effective as immediate PARP inhibition. The conclusion of the SOLO-2 analysis abstract by Frenel *et al.*[15] states, "the earlier use of olaparib remains optimal". The basis for this conclusion is uncertain. The marginal improvement in overall survival in SOLO-2 (upper limit of 95% confidence interval 1.00) is likely due to the fact only 38% of the placebo-treated patients were able to receive a PARP inhibitor[19]. More recently, the lack of overall survival benefit with niraparib in the Nova trial, which studied niraparib as second-line maintenance in BRCA mutated, HRD positive and HRD negative patients questions the importance of early PARP inhibitor therapy[20]. Although specific post clinical trial treatment was not collected in this study, it is postulated that many of the placebo-treated control patients subsequently received a PARP inhibitor because of their current greater availability commercially.

This emphasizes the need for a prospective randomized trial to compare the early use of a PARP inhibitor to the delayed use of a PARP inhibitor. In the design of this trial, one would have to think about when early PARP inhibitor therapy should occur. Numerous studies are suggested that 30% of BRCA mutated patients may not recur[21-23]. Therefore, is it reasonable from a quality of life, toxicity and cost perspectives to treat

Table 1. Studies of chemotherapy following PARP inhibitor therapy

Author Year	Study design	Patients	Results
Ang *et al.*[12] 2013	retrospective	Post olaparib platinum-based chemotherapy among BRCA mutated	48 patients RR 40%
Cecere *et al.*[13] 2020	retrospective	Post olaparib chemotherapy* among BRCA mutated	> 12 mo 14 platinum, 4 non-platinum RR 22% 6-12 mo 14 platinum, 13 non-platinum RR 11%
Baert *et al.*[14] 2020	retrospective	PD on 3rd platinum-based among BRCA mutated and nonmutated	No prior PARP (n = 57): PD 9% P = 0.003 Prior PARP (n = 35): PD 40%
Frenel *et al.*[15] 2020	SOLO-2	PFS following 3rd platinum-based chemotherapy among BRCA mutated	Olaparib (n = 54): 7 months Placebo (n = 42): 14.3 months
Rose *et al.*[16] 2021	retrospective	PFS following 2nd & 3rd platinum among BRCA mutated	2nd platinum No prior PARP (n = 108) 19.1 mo P = 0.005 Prior PARPi (n = 7) 8.0 mo 3rd platinum No prior PARP (n = 42) 18.4 mo P < 0.001 Prior PARPi (n = 13) 7.9 mo 2nd or 3rd platinum No prior PARP (n = 150) 19.1 mo P < 0.001 Prior PARPi (n = 20) 8.0 mo

PD: progressive disease; PFS: progression-free survival; *not able to associate platinum and response; PARP: poly-adenosine ribose polymerase.

100% of the BRCA mutated patients after first-line chemotherapy when only 70% of the patients may recur? Alternatively, would it be better to delay maintenance therapy once a patient has developed a recurrence?

As reported by Burger *et al.*[24], "Vascular endothelial growth factor and angiogenesis correlate directly with the extent of disease and inversely with progression-free survival and overall survival." Several antiangiogenic targeted therapies have been studied in ovarian cancer. The most widely studied antiangiogenic agent is bevacizumab. Two studies conducted in Europe (ICON 7) and in the United States Gynecologic Oncology Group (GOG) 218 demonstrated a benefit of maintenance bevacizumab in stage IV patients[25,24]. In ICON 7, patients were defined as high risk for progression, which included stage IV disease, inoperable stage III disease, or suboptimally debulked (> 1 cm) stage III disease. The addition of bevacizumab to chemotherapy and as maintenance for 12 cycles resulted in an improvement in overall survival [39.7 months *vs.* 30.2 months (HR 0.78, P = 0.03)][25]. For stage IV patients in GOG 218, the addition of bevacizumab to chemotherapy and maintenance improved the median survival by 10 months (42.8 months *vs.* 32.6 months, HR 0.75)[26]. However, no statistical benefit was seen in an exploratory analysis using ICON 7 criteria, suboptimal stage III and IV patients *vs.* all other stage III patients with survivals of 42.8 months *vs.* 45.6 months, respectively. The benefit of bevacizumab in the ICON 7 high risk for progression subgroup included inoperable stage III patients treated with neoadjuvant chemotherapy[25]. GOG 218 did not include patients treated with neoadjuvant chemotherapy and therefore cannot confirm or refute ICON 7 findings in this population.

Whether prior exposure to bevacizumab affects the response to subsequent platinum-based chemotherapy or platinum-based chemotherapy with bevacizumab has been evaluated in three randomized trials. In GO G 213, 10% of patients in each arm had received bevacizumab[27]. Despite prior bevacizumab, patients who were randomized to retreatment with bevacizumab had an improved progression-free survival, but this was not statistically significant as they comprised only 10% of the study population [HR 0.545 (0.292-1.017)]. In contrast, in the MITO16b/MANGO-OV2/ENGOT-ov17 trial, patients who received platinum-based chemotherapy with bevacizumab as part of first-line therapy were randomized to receive a platinum-based chemotherapy doublet either carboplatin and paclitaxel, carboplatin and gemcitabine or carboplatin and pegylated liposomal doxorubicin, with or without bevacizumab[28]. The median progression-free survival to

second-line platinum-based chemotherapy doublet was 8.8 months. The response rate for second-line platinum-based chemotherapy was dependent on the platinum-free interval, with different response rates for patients who progress at 6 to 12 months *vs.* those who progress after 12 months[4]. Table 2 is a list of platinum-based second-line chemotherapy regimens based on an increasing percentage of patients in the 6- to 12-month interval. GOG 213[27] and ICON 4[29] have a more favorable population of patients with a lower frequency of 6- to 12-month platinum-free interval patients 25% and 27%, respectively, compared to MITO16b/MANGO-OV2/ENGOT-ov17 with 35%. The MITO16b/MANGO-OV2/ENGOT-ov17 trial median progression-free survival of 8.8 months compares favorably to other platinum doublets in platinum-sensitive recurrent ovarian cancer. The median progression-free survival to second-line platinum-based chemotherapy doublet was not statistically different at 9.6 and 8.0 months, for patients who had progressed after bevacizumab or progressed on bevacizumab, respectively $P = 0.8$.

The AGO OVAR 2.21/NGOT- ov 18 trial, which compared carboplatin/gemcitabine/bevacizumab with carboplatin/pegylated liposomal doxorubicin/bevacizumab, allowed prior antiangiogenic therapy[36]. Prior antiangiogenic therapy was received in 47.5% of all patients, most commonly bevacizumab (87.3%). Prior antiangiogenic therapy did statistically decrease the median progression-free survival at 10.1 months (95%CI: 8.5-11.2) *vs.* 13.6 months (95%CI: 11.7-15.6) for carboplatin/gemcitabine/bevacizumab but not for carboplatin/pegylated liposomal doxorubicin/bevacizumab 11.3 months (95%CI: 10.1-13.8) *vs.* 14.4 months (95%CI: 12.3-16.8). The median overall survival was decreased more than 5 months with prior antiangiogenic therapy 25.1 months (95%CI: 19.3-27.8) *vs.* 30.7 months (95%CI: 27.4-36.2) for the carboplatin/gemcitabine/bevacizumab arm and by almost 10 months, 27.7 months (95%CI: 24.0-31.7) *vs.* 37.3 months (95%CI: 30.9-42.9) for the carboplatin/pegylated liposomal doxorubicin/bevacizumab arm, but these were not statistically significant.

Thirty-one percent of patients in each arm were in the 6-12 months platinum-free interval, so this progression-free survival is consistent with prior studies Table 2 and does not suggest any negative impact of prior antiangiogenic therapy.

RADIATION CONSOLIDATION

Ovarian cancer is radiosensitive, but the potential for metastasis throughout the abdominal cavity requires whole abdominal radiation. While bulky residual disease is difficult to treat, it was believed that patients who had undergone cytoreductive surgery and platinum-based chemotherapy, with the potential for further chemical cytoreduction, consolidation with whole abdominal radiation might be more effective. Lawton *et al.*[37] from the West Midlands Ovarian Cancer Group randomized patients to whole abdominal radiation therapy using a moving strip technique or one year of chlorambucil. Overall survival at two years was 35%, with no significant difference in survival between the two groups despite the fact that approximately 50% of the patients were optimally debulked prior to consolidation. Toxicity was considerable in both arms, and almost 50% of patients were unable to complete the planned treatment in both arms. A subsequent British trial by the North Thames Group evaluated consolidation platinum-based chemotherapy *vs.* whole abdominal radiation[38]. One hundred seventeen patients with residual disease of 2 cm or less at second-look laparotomy or laparoscopy were then randomized to receive consolidation therapy, either five further courses of carboplatin at the same dosage or whole-abdominal RT (24 Gy). The median survival for the whole group from the date of surgery was 25 months. No statistical difference was found in either survival or disease-free survival between those patients who received consolidation chemotherapy and those who were treated with abdominal RT. The Swedish-Norgewian Ovarian Cancer Study Group performed an observation controlled trial comparing receive whole-abdominal irradiation (WAR) or six courses of consolidation chemotherapy (cisplatin 50mg/m^2 and doxorubicin 50mg/m^2 or

Table 2. Chemotherapy only arms from randomized trials of platinum-based chemotherapy in platinum-sensitive ovarian cancer

Study	GOG 213[27]	ICON 4[29]	MME[28]	Calypso[30]	Calypso[30]	02004093*[31]	AGO 2.2[32]	Oceans[33]	00318370*[34]	00170677*[35]
Invest Agent Regimen	**Bev P C**	**None P C**	**Bev P C**	**None PLD C**	**None P C**	**Pertuzumab P C & G C**	**None G C**	**Bev G C**	**Farletuzumab P C**	**None T C**
6-12 mo	25%	27%	35%	35%	36.1%	38.7%	39.9%	42.1%	53.3%	64%
> 12 mo	75%	73%	65%	65%	63.9%	61.3%	59.8%	57.9%	46.7%	36%
PFS	10.4 mo	12 mo	8.8 mo	11.3 mo	9.4 mo	8.7 mo	8.6 mo	8.7 mo	9.0 mo	10 mo

NCT number*; Bev: bevacizumab; C: carboplatin; G: gemcitabine; MME: MITO16b/MANGO-OV2/ENGOT-ov17; P: paclitaxel; PLD: pegylated liposomal doxorubicin; T: topotecan; GOG: Gynecologic Oncology Group.

epirubicin 60mg/m^2) *vs.* no further treatment[39]. Seven hundred and 42 patients were prospectively studied. Following primary cytoreductive surgery followed by induction chemotherapy (four courses of cisplatin 50mg/m^2 and doxorubicin 50mg/m^2 or epirubicin 60mg/m^2), patients with no evidence of disease (NED), a complete clinical response (cCR), or partial response (cPR) underwent a second-look laparotomy. At second-look laparotomy, patients were classified as having a pathologic complete response, microscopic residual disease, or macroscopic residual disease. One hundred and seventy-two patients with a pathologic complete response or microscopic residual disease entered into the randomized study. Patients with a pathologic complete response who received radiation therapy had improved progression-free survival compared to patients who received chemotherapy or observation (P = 0.032), but overall survival was not improved (P = 0.084). Subsequent post-study treatment was not collected or reported in this trial. Preclinical data suggests a possible interaction between radiation resistance and chemotherapy resistance[40]. Since whole abdominal radiation is not widely utilized clinically, a clinical correlation has not been documented in the literature.

Lastly, although Intraperitoneal P32 was not found to be an effective consolidation therapy for stage III ovarian cancer patients after negative second-look laparotomy, it did not adversely affect overall survival[41].

MAINTENANCE CHEMOTHERAPY

In 2003, Markman reported the results of the SWOG/GOG 178 trial, which demonstrated that following first-line chemotherapy, patients randomized to 12 cycles of paclitaxel *vs.* three cycles of paclitaxel had an improved progression-free survival (28 months *vs.* 21 months $P < 0.005$)[42]. Once this progression-free survival improvement was reported, patients in the 3 cycle arm were offered additional therapy. Possibly, as a result of this delayed crossover, there was no difference in overall survival. Conte *et al.*[43] performed a trial of six cycles of paclitaxel *vs.* observation following primary chemotherapy. No difference in overall survival was noted. The Gynecological Oncology Group performed a larger confirmatory trial (GOG 212) comparing 12 cycles of paclitaxel *vs.* 12 cycles of polyglutamate paclitaxel (Xyotax) *vs.* observation[44]. No difference in overall survival was noted at 51.3 months, 60.0 months, and 54.8 months for paclitaxel, Xyotax, and observation, respectively. Other studies have used topotecan[45,46] or epirubicin[47] as maintenance therapy *vs.* observation, but no difference in

overall survival has been noted. In summary, although multiple chemotherapy agents have been studied as maintenance therapy and ovarian cancer, it is clear that they have not improved overall survival. When non-platinum maintenance chemotherapy was utilized, since patients were off platinum, the platinum-free interval was not affected, and anecdotally subsequent chemotherapy was not adversely affected by non-platinum maintenance chemotherapy.

In summary, patients with advanced-stage ovarian cancer have high responses to primary therapy but have a high recurrence rate. Multiple studies have looked at the use of maintenance or consolidation therapy to prevent recurrence. Currently, both the use of bevacizumab and PARP inhibitors are favored as possible maintenance therapies. Prior use of bevacizumab does not appear to adversely affect subsequent response to platinum-based chemotherapy or platinum-based chemotherapy with bevacizumab. Since the response to PARP inhibitors is related to platinum sensitivity, it suggests a common mechanism of resistance. Prior therapy with PARP inhibitors induces platinum resistance to subsequent platinum-based therapy and negates classic predictors of response such as platinum-free interval and BRCA mutational status. Randomized trials are needed to determine the best time to initiate PARP inhibitor therapy.

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Cancer Drug Resistance

Review

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Ovarian cancer recurrence: is the definition of platinum resistance modified by PARPi and other intervening treatments? The evolving landscape in the management of platinum-resistant ovarian cancer

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Abstract

Definitions of platinum resistance have been questioned and changed over the last five years, even though no predictive biomarker of resistance exists. These have sculpted how we approach platinum retreatment and, consequently, how we devise new treatment strategies for those patients with tumour progression on platinum therapy. Platinum-non-eligible ovarian cancer is treated with single-agent non-platinum drugs. When bevacizumab can be added to chemotherapy, progression-free survival improves significantly. For patients with a BRCA mutation, PARP inhibitor monotherapy is an option compared to chemotherapy. There is currently no clearly identified role for immune-checkpoint inhibition in this patient population. This review describes some of the challenges in treating patients with platinum resistance and suggests refinements in the selection of patients most likely to benefit from targeting a DNA damage response, angiogenesis or immune modulation. It also describes novel agents of interest and possible mechanisms of the synergy of therapeutic combinations.

Keywords: Platinum resistance, PARP inhibitors, VEGF inhibitors, immune checkpoint inhibitors, DNA damage response

INTRODUCTION

Background of platinum retreatment

The idea of platinum rechallenge was introduced in the 1980s at a moment in history when few treatments were available for recurrent ovarian cancer. Across several Phase II studies, the treatment-free interval (TFI) was one of the most important variables predicting response to second-line chemotherapy[1]. Later, Markman and Hoskins proposed that trials of new agents be stratified into primary platinum-resistant, secondary platinum-resistant, potentially platinum-sensitive and those with indeterminate sensitivity[2]. These definitions underwent further refinement, with variation in the cut-offs of TFI between 4 and 12 months for intermediate platinum-sensitive disease and ultimately 6 months for being considered as platinum-sensitive, with this latter definition being used for the next 3 decades[3]. It was first rigorously questioned at the 2010 Gynecologic Cancer InterGroup (GCIG) Ovarian Cancer Consensus meeting, during which its use was criticized as the response to platinum gradually increases with TFIp (TFI after platinum) in a non-linear way[3]. During the fifth GCIG consensus meeting in 2015, the terminology: platinum-sensitive and platinum-resistant in clinical trials was replaced with TFIp considered as a continuous variable among others discussed below.

Current definitions of platinum resistance and clinicopathological predictors of platinum responsiveness

According to ESMO-ESGO consensus meeting guidelines for the management of recurrent ovarian cancer[4], platinum-non-eligible ovarian cancer (PNEOC) patients are those who progress on or immediately after their last platinum-based chemotherapy or have contraindications to platinum. Platinum-eligible ovarian cancer (PEOC) includes all other cases of relapse. This includes patients without evaluable or no residual disease after primary surgery or who have relapsed following stage I disease.

There is no biomarker of platinum resistance. However, research is ongoing to define predictive biomarkers of resistance as well as prognostic markers that may be used as tools to guide treatment selection in patients with PNEOC.

For example, Lee *et al.*[5] have developed a nomogram to refine prognostication in this group using six pre-treatment variables [TFIp, performance status, size of the largest tumour, cancer antigen-125 (CA-125), haemoglobin and the number of metastatic organ site]. This nomogram improved overall survival prediction in patients with PEOC compared to models with fewer prognostic factors or TFIp alone. This could have applications for stratification in clinical trials and counselling patients.

An important predictive variable of response to platinum is tumour biology and histology; for example, response rates are lower in patients with clear cell, low-grade serous and mucinous ovarian cancers[6]. Tumour molecular changes, including the presence of homologous recombination deficiency, increase the likelihood of a response to platinum[7].

Mechanisms of platinum resistance

DNA damage response detection and repair

The DNA damage response is utilised to detect DNA damage and initiate DNA repair in order to maintain genomic integrity[8]. It consists of a network of interrelated signalling pathways, which can be broadly divided into homologous recombination (HR) dependent and HR independent repair pathways [Figure 1].

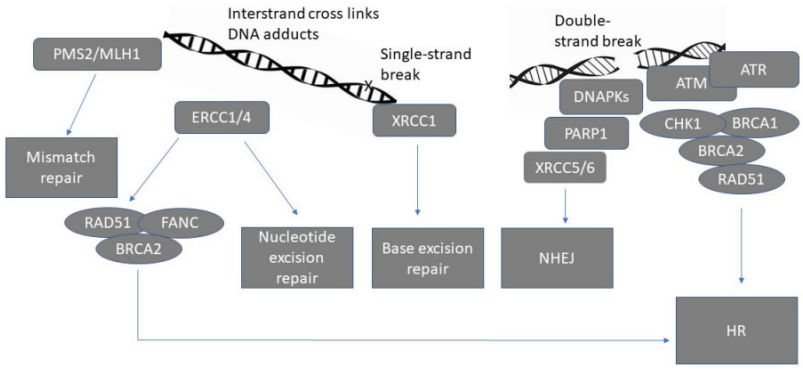

Figure 1. Schematic representation of major DNA repair pathways[8]. PMS2: postmeiotic segregation increased 2, MLH1: mutL homolog 1, ERCC1/4: Excision repair cross-complementing 1/4, RAD51: RAD51 recombinase, FANC: Fanconi anaemia complementation group, BRCA1/2 : Breast cancer gene 1/2, DNAPKs: DNA protein kinases, PARP1: Poly (ADP-ribose) polymerase, XRCC5/6: X-Ray Repair Cross Complementing 5/6, ATM: ATM Serine/Threonine Kinase, ATR: Ataxia telangiectasia and Rad3-related, CHK1: checkpoint kinase 1, HR: homologous recombination, NHEJ: non-homologous end joining.

HR dependent repair is designed to repair double-strand DNA breaks and interstrand crosslinks and, to a lesser extent, other kinds of DNA damage[9]. DNA repair, which is not dependent on HR, includes non-homologous end joining (NHEJ) for interstrand, double-strand breaks and intrastrand breaks. Other HR independent pathways that are less error-prone include mismatch repair, base excision repair and nucleotide excision repair (NER). These are typically recruited for the repair of single-strand breaks or damage induced by DNA adducts[10].

The ERCC1-XPF nuclease enzyme is translated from the mRNAs of the ERCC1 and ERCC4 genes[11]. This enzyme plays a key role in DNA damage repair chiefly via NER and also that caused by interstrand crosslinks and double-strand breaks by HR and NHEJ[11]. ERCC1-XPF targeting is a strategy being explored to increase the sensitivity of cancer cells to some DNA-damaging chemotherapeutic agents.

The master sensors (ATM, ATR, and DNA-PKs) are large kinases that sense DNA damage and initiate repair signalling cascades by phosphorylating key proteins, which are primarily involved in HR dependent repairs such as BRCA1, CHK1, CHK2, and RAD51[12]. The activation of signalling transduction pathways, including PI3K/AKT, promotes the activation of DNA damage response (DDR) cell checkpoints that halt cell cycle progression, allowing more time for DNA repair[8].

Correlation between platinum sensitivity and PARPi (PARP inhibitor) sensitivity

BRCA-deficient ovarian cancers have increased platinum sensitivity[13-14]. *In vitro,* reversion of BRCA mutations confers platinum and PARPi resistance[15-16]. In clinical studies, response to olaparib correlates with TFIp[17]. There is also a correlation between deficiency in other HR genes, *ex vivo* PARPi sensitivity, and platinum sensitivity in patients[18]. Multiple resistance mechanisms to platinum and PARPi have been described independently, although, following on from the above, significant mechanistic crossover exists.

Mechanisms of platinum and PARPi resistance

HR-dependent mechanisms of resistance include restoration of BRCA function by secondary or reversion mutations, or restoration of HR by loss of 53BP1, RIF1 or the shieldin complex amongst others [Table 1]. One major limitation of standard HR assays is that they are mostly insensitive to the detection of homologous recombination deficiency (HRD) reversion[16]. HR functional assays require viable cancer cells to be exposed to DNA damaging agents *ex vivo*, which therefore limits the access to samples and assay

Table 1. Mechanisms of PARP/platinum resistance

Mechanism	Proteins involved
PARP activity alteration loss of PARG increased stabilisation of replication forks	RAS PI3K/AKT PARP
Altered ion channel drug accumulation upregulation of drug efflux pumps Intracellular drug inactivation	VRAC MRP 2
Restoration of BRCA function through secondary reversion mutation modification of other HR proteins	BRCA1/2 53BP1 RIF1 shieldin complex

RAS = Rat sarcoma, PI3K phosphor-inositol 3-kinase; AKT: AK strain transforming; PARP: Poly (ADP-ribose) polymerase; PARG: Poly ADP (adenosine diphosphate) Ribose Glycohydrolase; VRAC: volume-regulated anion channel; MRP2: multidrug resistance-associated protein 2; BRCA1/2: Breast cancer gene 1/2; 53BP1: Tumour Protein P53 Binding Protein 1; RIF1: Replication Timing Regulatory Factor 1.

reproducibility.

Mechanisms independent of HR include increased stabilisation of replication forks, upregulation of drug efflux pumps, PARP activity alteration, loss of PARG and RAS/PI3K/AKT pathway activation. Associated overexpression of STAT5B and RELA, two transcription factors associated with platinum resistance, is less well understood[19].

Platinum resistance may also emerge due to reduced intracellular drug accumulation, for example, through reduced intracellular drug uptake, intracellular drug inactivation, enhanced DNA repair or altered apoptotic signalling pathways[20].

Refining biomarkers of resistance to platinum and PARPi

HRD is a useful biomarker for predicting the initial response to both platinum chemotherapy and PARPi, though biomarkers of resistance require much refinement.

Standard tests for HRD, including the Myriad genomic instability score and Foundation Medicine loss of heterozygosity test, predict the presence of HRD based on genomic features[21]. These and other genomic tests vary in terms of the genomic features measured and the threshold definitions for identifying patients considered to have HRD. Clinically, HRD test results and PARPi responses can be discordant. This may be because tumours with reversion mutations that restore HR function still exhibit evidence of HRD on these assays or that alternative HR independent PARPi resistance mechanisms may be playing a predominant role. Functional assays of HR genes may overcome some of these challenges in predicting the presence of HRD[21]. The measurement of somatic mutations, such as a BRCA reversion mutation in ctDNA, is non-invasive and warrants further development[22].

Approximately 40% of high-grade serous HR proficient ovarian tumours, demonstrate increased Cyclin E expression by CCNE1 gene amplification, increased copy numbers or enhanced protein expression[23]. These CCNE1 high tumours are associated with platinum resistance and poor survival[24].

Other tumour factors contributing to treatment resistance

Tumour microenvironment

Together with genomic alterations in the DNA damage response, the tumour microenvironment is an increasingly recognised contributor to our understanding of resistance mechanisms in ovarian cancer[19].

The increased infiltration of immunosuppressive regulatory T cells has been correlated with enhanced tumour growth[25], whereas the presence of CD8+ tumour infiltrating lymphocytes is correlated with enhanced survival[26]. The most predominant immune cells associated with ovarian cancer are macrophages. Tumour-associated macrophages (TAMs) are easily polarised by tumour-cell-producing colony-stimulating factor-1 into an immunosuppressive M2-like phenotype[27]. The main pro-tumoural function of M2-like TAMs is the secretion of cytokines and exosomes that induce microRNAs, which directly promote the survival, invasion potential and chemoresistance of ovarian cancer cells[27]. PD-1, PD-L1 expression and Tumour Mutational Burden have not shown consistent validity as predictive biomarkers for immune checkpoint inhibition in ovarian cancer[19]. Retinoic acid-inducible gene-I overexpression is correlated with platinum-resistant ovarian and other refractory cancers[28]. Its overexpression is associated with local immunosuppressive changes and a distinct immune signature. Extensive stromal desmoplasia has also been associated with platinum resistance[29].

Altered metabolism in cancer tissues

Accumulating evidence suggests that tumour metabolism differs from that of matched normal tissues[30], and metabolic reprogramming may cause therapy resistance. Of relevance to platinum resistance, in one cisplatin-resistant PDX ovarian cancer model glycolysis, the tricarboxylic acid and urea cycle pathways were deregulated with higher mitochondrial respiration. This may suggest a role for therapies that modulate metabolism, such as metformin. Other drugs used in non-cancer indications and new small molecule inhibitors of mitochondrial complexes are being increasingly utilised to target cancer metabolism[31].

CURRENT APPROACHES TO THE MANAGEMENT OF PNEOC

Chemotherapy

Highlighting the clinical relevance of the arbitrariness of TFIp to decide on subsequent platinum retreatment, Lindemann *et al.*[32] compared second-line platinum *vs.* non-platinum regimens in a cohort of patients who would have traditionally been regarded as platinum-resistant, i.e., those with a TFIp < 6 mo. They found a greater CA-125 response rate of 51 *vs.* 21% ($P < 0.001$) in those treated with a platinum-based therapy compared to a non-platinum regimen; and in those patients with TFIp between 3 and 6 months, improved overall survival.

Using the new and modified definition of resistance, patients with PNEOC, i.e., those progressing on platinum, are typically offered non-platinum-based chemotherapy such as weekly paclitaxel, pegylated liposomal doxorubicin (PLD) or topotecan with or without bevacizumab[3]. There have been comparatively few randomised phase III trials in this setting. Table 2 summarises the key data. In the CORAIL trial, comparing lurbinectedin to PLD or topotecan in patients with a TFI < 6 mo, the PFS was similar across all groups[33]. In the AURELIA trial, patients who relapsed after 1-2 prior lines of platinum were randomised between topotecan, PLD, or weekly paclitaxel with or without bevacizumab[34]. The combination of bevacizumab with PLD, weekly paclitaxel or topotecan improved mPFS compared to chemotherapy alone. Alternative non-platinum options include oral etoposide, tamoxifen, gemcitabine and treosulfan[3].

PARP-inhibitors

There is a role for single-agent PARP inhibitors, particularly in those with BRCA mutations that have become resistant to platinum. This is demonstrated in the single-arm Phase II QUADRA trial, in which patients were treated with niraparib after more than three lines of therapy that did not include a previous PARPi. The clinical benefit rates in patients with a BRCA1 or two mutations and TFIp < 6 months were 38% and 33%, and in the group that was platinum-refractory, 50% and 31%, at 16 and 24 weeks, respectively[35]. Although this is a single-arm Phase 2 study, the data does suggest an important role for niraparib in

Table 2. Phase III trials in PNEOC

Trial	Treatment Arms	mPFS	Reference
CORAIL	Lurbinectedin *vs.* control arm (PLD *vs.* topotecan)	3.5 *vs.* 3.6 mo HR = 1.057 *P* = 0.6294	Gaillard *et al. (2018)*[33]
ARIEL4	Rucaparib *vs.* weekly paclitaxel (TFIp 1-6 months)	6.4 *vs.* 5.7 mo HR = 0.78 (95%CI: 0.54-1.13)	*Oza (2021)*[36]
AURELIA	Bevacizumab plus chemotherapy (PLD or topotecan or weekly paclitaxel) *vs.* chemotherapy alone	6.7 *vs.* 3.4 mo HR = 0.48 *P* < 0.001	Pujade-Lauraine *et al. (2014)*[34]
JAVELIN Ovarian 200	Avelumab plus PLD *vs.* PLD *vs.* Avelumab	3.7 *vs.* 3.5 *vs.* 1.9 mo HR (combination *vs.* PLD) = 0.78 one-sided *P* = 0.03 HR (avelumab *vs.* PLD) = 1.68 one sided *P* =0.99	Pujade-Lauraine *et al. (2021)*[41]

PLD: pegylated liposomal doxorubicin; mPFS: median progression-free survival; mo: months; HR: hazard ratio; TFIp: platinum-free interval; CI: confidence interval.

PNEOC patients with a BRCAm. ARIEL 4 (NCT02855944) is a Phase 3 study evaluating rucaparib *vs.* standard of care chemotherapy in patients with BRCA-mutated, relapsed ovarian cancer. Approximately half of the patients included in the trial had a TFIp of between 1 and 6 months, and the mPFS in this group was 6.4 months for rucaparib and 5.7 months for chemotherapy (HR 0.78, 95% CI 0.54-1.13)[36].

VEGF inhibitors

Angiogenesis is a hallmark of cancer[30], with neo-angiogenesis abundantly present in ovarian cancer. Antiangiogenic therapy plus chemotherapy has shown an improvement in responses and PFS in PEOC compared to chemotherapy alone[3], and improvements in PFS have also been demonstrated in patients with a TFIp < 6 months in the Phase III AURELIA trial using the VEGF-A monoclonal antibody, bevacizumab, plus chemotherapy[34], or in smaller randomised Phase II trials of VEGF-R small molecule inhibitors, pazopanib with weekly paclitaxel (MITO-11)[37] or sorafenib with topotecan (TRIAS)[38].

In AURELIA, the combination of bevacizumab with PLD, weekly paclitaxel or topotecan improved mPFS compared to chemotherapy alone in patients who relapsed after 1-2 prior lines of platinum[34] [Table 2]. However, it remains unclear based on these data when it might be appropriate to stop chemotherapy in those continuing to respond to the combination.

Immune checkpoint inhibitors

The results of trials of Immune checkpoint inhibitors (ICPI) monotherapy in ovarian cancer have been disappointing. In two Phase II trials of programmed cell death protein-1/ligand-1 (PD-1/PD-L1) inhibitors, pembrolizumab and avelumab showed little benefit in ovarian cancer cohorts[39-40]; however, it was hoped that in subgroups of patients including PNEOC patients, they may have a niche role.

Avelumab, either alone or in combination with PLD in platinum-resistant ovarian cancer (JAVELIN Ovarian 200), failed to show a significant OS benefit compared to PLD alone [Table 2]. However, exploratory analyses suggest there may have been a benefit of the combination in those with an initial response to earlier lines of chemotherapy[41]. As a role for bevacizumab has been demonstrated in AURELIA, the question of whether ICPI enhances this benefit is relevant. NRG-Gy009 study

(NCT02839707), which has completed recruitment, compared the combinations of PLD and bevacizumab *vs.* PLD and atezolizumab *vs.* PLD and atezolizumab and bevacizumab; the results are awaited [Table 3].

Combining two ICPIs, such as anti-PD-1 and anti-cytotoxic T lymphocyte-associated-4 antibodies, may increase the activity of immunotherapy with evidence that nivolumab and ipilimumab showed a longer PFS than nivolumab alone (mPFS of 3.9 *vs.* 2 months), albeit with greater toxicity[42]. However, these figures are notably comparable to those seen for single-agent non-platinum-based chemotherapies.

Other trials are exploring the use of maintenance immunotherapy after chemotherapy to improve PFS. One such study is the PROMPT Phase II trial, in which patients receive pembrolizumab after 4-6 cycles of weekly paclitaxel (NCT03430700).

NEWER STRATEGIES FOR OVERCOMING PLATINUM RESISTANCE

The above data show that with current regimens, mPFS is short, and tools to select patients likely to benefit most are required.

Refinements in patient selection for bevacizumab

There are currently no predictive biomarkers for bevacizumab response available in the clinic. Angiogenic markers, including micro-vessel density, CD31 expression and tumour VEGF-A levels, may provide prognostic information in recurrent ovarian cancer. These were identified in the Gynecologic Oncology Group (GOG) 218 study as potential predictive biomarkers for the use of bevacizumab[43]. Another retrospective analytical study showed that a signature comprising alpha-1 acid glycoprotein, mesothelin, FLT4 and CA-125 identified those patients more likely to benefit from bevacizumab[44]. In a concordance exploratory study of ICON7 samples, plasma concentrations of several angiogenesis-associated factors were determined using multiplex ELISAs, with high Ang1 and low Tie2 levels correlating best with PFS.

Tie1 and 2 are receptor tyrosine kinases that function as key regulators of blood vessel development and pathological processes including angiogenesis[45]. One observational biomarker study (VALTIVE) is currently recruiting to determine the clinical value of measuring plasma Tie2 concentrations in ovarian cancer patients who are receiving bevacizumab (NCT04523116).

Novel treatments

Targeting Ataxia telangiectasia and Rad3-related

Targeting Ataxia telangiectasia and Rad3-related (ATR) is an important kinase regulating the DDR. It is responsible for sensing replication stress and signalling to cell cycle checkpoints to initiate repair[46]. ATR inhibitors have been shown to reduce the rate of DNA repair in cells, thereby increasing DNA damage and apoptosis[47]. Single-agent ATR inhibition appears to show some efficacy in PNEOC[46].

G-Quadruplex (G4) stabilisation

G4 structures can form at thousands of sequences in the human genome and increase the propensity for DNA damage by impeding DNA polymerase, and thereby DNA damage repair processes[48]. CX-5461 is a small molecule RNA polymerase transcription inhibitor that selectively kills HR deficient cancer cells by stabilising G4 structures[49]. Phase 1 studies of CX-5461 are being investigated in solid tumours, including in a platinum/PARPi resistant ovarian cancer cohort (NCT04890613).

Cell cycle checkpoint inhibition

The cell cycle checkpoint regulators CHK1 and CHK2 halt cell division to allow DNA damage to be

Table 3. Combination trials of interest

clinicaltrials.gov identifier	Treatment arms	Proposed mechanism of synergy
NCT02502266 (NRG-Gy005)	Olaparib *vs.* cediranib *vs.* olaparib-cediranib *vs.* investigator's choice of chemotherapy (paclitaxel/topotecan/PLD).	antiangiogenic therapy induces a hypoxic tumour microenvironment, thereby enhancing synthetic lethality by downregulation of HR genes
NCT02839707 (NRG-Gy009)	PLD and bevacizumab *vs.* PLD and atezolizumab *vs.* PLD and atezolizumab and bevacizumab	VEGF targeting reduces inhibition of tumour immune cell suppression which permits increased efficacy of PD-L1 inhibition and chemotherapy cytotoxicity
NCT04065269 (ATARI)	ceralasertib and olaparib *vs.* ceralasertib	ATR plus PARP inhibition overcomes PARPi resistance by inducing increases in replication fork stalling, double-strand breaks, and apoptosis

PLD: pegylated liposomal doxorubicin; HR: homologous recombination; ATR: Ataxia telangiectasia and Rad3-related; PARP: Poly (ADP-ribose) polymerase; PARPi: PARP inhibitor.

repaired before DNA replication[50]. Cell cycle checkpoint inhibition may thereby prevent the progression of cancer cells through the cell cycle, halting replication and tumour progression. WEE-1 inhibitors block the activity of WEE-1 kinase, a G2 cell-cycle checkpoint, and enhance cancer cell apoptosis[51].

Prexasertib is one example of a CHK1 inhibitor, which demonstrated responses in a phase II trial that were most marked in patients with platinum-resistant ovarian cancer[52]. A phase II study of the combination of AZD1775, a WEE-1 inhibitor and carboplatin in platinum-resistant ovarian cancer, demonstrated an ORR of 43%[53].

Epigenetic re-sensitisation

Treatment resistance is often associated with the accumulation of epigenetic changes[54]. It has therefore been hypothesised that epigenetic modulation may re-sensitise tumours to platinum chemotherapy.

The DNA methyltransferase (DNMT) and Histone Deacetylase inhibitors have shown little activity as single agents in platinum-resistant ovarian cancer. However, in combination, they may enhance sensitivity to platinum by altering epigenetic regulation of gene expression. In a randomised phase II study assessing the DNMT inhibitor, guadecitabine in combination with carboplatin *vs.* investigator's choice of chemotherapy, the PFS rate at 6 months was 37% *vs.* 11%[55]. The DNA damage initiated by DNMT inhibitors is repaired by the BER pathway, in which PARP1 plays a key role, and therefore there may also be a rationale to combine DNMT and PARP inhibition.

Combination approaches

Angiogenesis and PARPi

Antiangiogenic therapy has been shown to induce a hypoxic tumour microenvironment associated with the downregulation of HR genes[56], providing the rationale to enhance the synthetic lethality of PARPi with angiogenesis inhibitors which also separately work to interfere with angiogenesis.

The Phase II AVANOVA2 trial compared the combination of niraparib and bevacizumab *vs.* single-agent niraparib in patients with PEOC[57]. Niraparib plus bevacizumab significantly improved mPFS compared with niraparib alone (11.9 mo *vs.* 5.5 mo; HR 0.35, $P < 0.0001$) and has provided a rationale to test this strategy in PNEOC.

EVOLVE was a phase II trial of cediranib-olaparib in ovarian cancer progressing on PARPi, recruiting a cohort of patients who were also defined as platinum-resistant, with 2/10 patients in this cohort

demonstrating a PR[58]. The anti-tumour activity of this combination continues to be assessed in the randomised Phase III NRG-Gy005 (NCT02502266) trial currently recruiting patients with platinum-resistant ovarian cancer to receive either olaparib, cediranib, olaparib-cediranib or investigator's choice of chemotherapy (paclitaxel/topotecan/PLD) [Table 3].

DDR response

In a large panel of acquired and *de novo* PARPi- and platinum-resistant CCNE1 amplified *in vitro* and PDX models, ATR and PARPi synergy was demonstrated[59]. This, amongst other data, has provided the rationale for the combination of ceralasertib and olaparib for recurrent platinum-resistant ovarian cancer in CAPRI[60]. Although no objective responses were demonstrated, the combination was well tolerated, and in two patients with BRCA1 mutations, a 50% fall in CA-125 was seen.

It will be interesting to see the data from the combination arm of ATARI (NCT04065269) in platinum-resistant ovarian clear cell cancer and carcinosarcomas, which may provide insights into how the combination of these drugs may alter the DDR in those subgroups of patients that do not classically display responsiveness to chemotherapy and PARPi [Table 3].

There is a clear rationale to combine other drugs regulating the DDR described earlier in this review, for example, WEE1 inhibitors, with platinum-based chemotherapy and PARPi.

Immunotherapeutic combinations

An alternative strategy is the combination of an ICPI with a PARPi, chemotherapy or other DDR modifying drugs. One possible mechanism of synergy is the observation that PARPi can activate the STING (stimulator of interferon genes) pathway to increase T-cell tumour infiltration[61]. TOPACIO was a Phase 1/2 trial testing niraparib plus pembrolizumab in platinum-resistant ovarian and triple-negative breast cancer patients. A subgroup analysis of the ovarian cancer cohort showed that the combination was promising for patients without HR deficiency[62]. There was a small cohort of patients in this group, and therefore other larger studies will need to focus on immunogenomic profiling to select patients most likely to benefit from this strategy.

CONCLUSION

Although the definition of true resistance to platinum-based chemotherapy has changed over the last four decades, few treatments have significantly changed outcomes in the vast majority of patients in this cohort. Next-generation sequencing has become faster and more affordable due to automation, which is permitting standardisation of techniques for analysing liquid biopsies and immunogenomic profiling. These refinements may lead to an improvement in patient selection for some of the novel strategies and combinations discussed in this review. Biomarker-driven trial designs will accelerate the better selection of and sequencing of treatment lines, including those targeting immune modulation, modification of the DNA damage response and angiogenesis inhibition.

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Not applicable.

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Cancer Drug Resistance

Review

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Fusobacterium nucleatum: a new player in regulation of cancer development and therapeutic response

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Abstract

A dysbiosis in microbial diversity or functionality can promote disease development. Emerging preclinical and clinical evidence emphasizes the interplay between microbiota and both disease evolution and the treatment response of different cancers. One bacterium that has garnered much attention in a few cancer microbiota studies is *Fusobacterium nucleaum* (Fn). To provide updated knowledge of the functional role of Fn in cancer prevention and management, this review summarizes the relationship among Fn, cancer, and chemoimmunotherapy response, with the potential mechanisms of action also intensively discussed, which will benefit the development of strategies to prevent or treat cancer via Fn-based therapeutic interventions.

Keywords: *Fusobacterium nucleatum*, tumor microenvironment, immune evasion, metastasis, chemoresistance

INTRODUCTION

Cancer, as the second leading disease-related cause of death in humans worldwide, affects almost all body regions[1]. Currently, cancer progression and resistance to therapy remain major challenges in cancer treatment and the main causes of poor prognosis[2]. Among the factors that contribute to cancer development and chemotherapy response, in addition to host genetic susceptibility and environmental exposures, a new and important player is emerging in regulating the development and drug resistance of cancer: microbiota[3].

The conventional paradigm proposes that a balanced microbiota is positively health-associated, while damage in microbial diversity or functionality, including dysbiosis or unbalanced microbiota, can promote the development of disease, such as various cancers[3-5]. Emerging preclinical and clinical evidence links the microbiota and their metabolites with carcinogenesis[6]. Related studies revealed that cancer formation can be driven by microbial pathogens in 15%-20% of cancer cases[7]. Bacteria *Prevotella gingivalis*, *Helicobacter pylori*, *Salmonella typhi*, *Prevotella melaninogenica*, *Chlamydia pneumoniae*, *Streptococcus mitis*, *Streptococcus bovis*, and *Capnocytophaga gingivalis* may cause different types of cancers in humans[8-12]. Importantly, recent insights shed light on the influence of the microbiota on the response to chemotherapy. The interplay between the microbiota and both tumor evolution and the treatment response of different cancers, especially colorectal cancer (CRC), has recently been studied[13]. One bacterium that has garnered wide interest in a few cancer-related microbiota studies is *Fusobacterium nucleatum* (Fn). Although Fn has been considered as an opportunistic pathogen for infections, its role as a cancer- or chemoresistance-causing member is revealed in various ways, by which Fn contributes to cancer initiation, progression, and the response to chemotherapy.

Based on these findings, we undertook a systematic review of the role of Fn as an oncobacterium. The research included in this review covers a period of 20 years, until the end of November 2021.

THE ROLE OF THE FN IN CARCINOGENESIS

Fn is a Gram-negative anaerobic bacillus that exists, among others, in the human oral cavity and the gastrointestinal tract. It exerts pro- or anti-pathogenic effects in the oral cavity affecting human periodontal health and diseases. The high abundance of Fn has been reported to be associated with head and neck cancer, esophageal cancer, pancreatic cancer, prostatic cancer, cervical carcinoma, and breast cancer [Figure 1][14-18]. Some investigators proposed that Fn is a passenger that multiplies under favorable conditions induced by malignant tumors[19]. However, more research data support Fn as having a mechanistic role in driving carcinogenesis rather than being a microbial passenger, whose effects can be classified into three steps: initiation, promotion, and progression[20]. In the following section, we review studies on the mechanisms by which Fn initiates and promotes carcinogenesis and enhances disease progression.

The first step: adhering to and invading human epithelial and endothelial cells

Fn accumulates in distant organ

Adherence and invasion are essential mechanisms for oncobacterium colonization, dissemination, and evasion, subsequently inducing a series of host responses. As one of the most abundant bacteria in the oral cavity, Fn can cause infectious inflammatory conditions at multiple body sites in addition to inflammation of the gingival tissue[21-24]. It has also been proved that mislocalization of Fn is associated with many cancers. Although it seems possible that Fn directly spreads from the oral cavity to the colon, certain evidence demonstrates that Fn reaches distant sites of inflammation and tumorigenesis via a hematogenous route[25,26]. However, how can an oral bacterium be implicated in so many infections and cancers within and

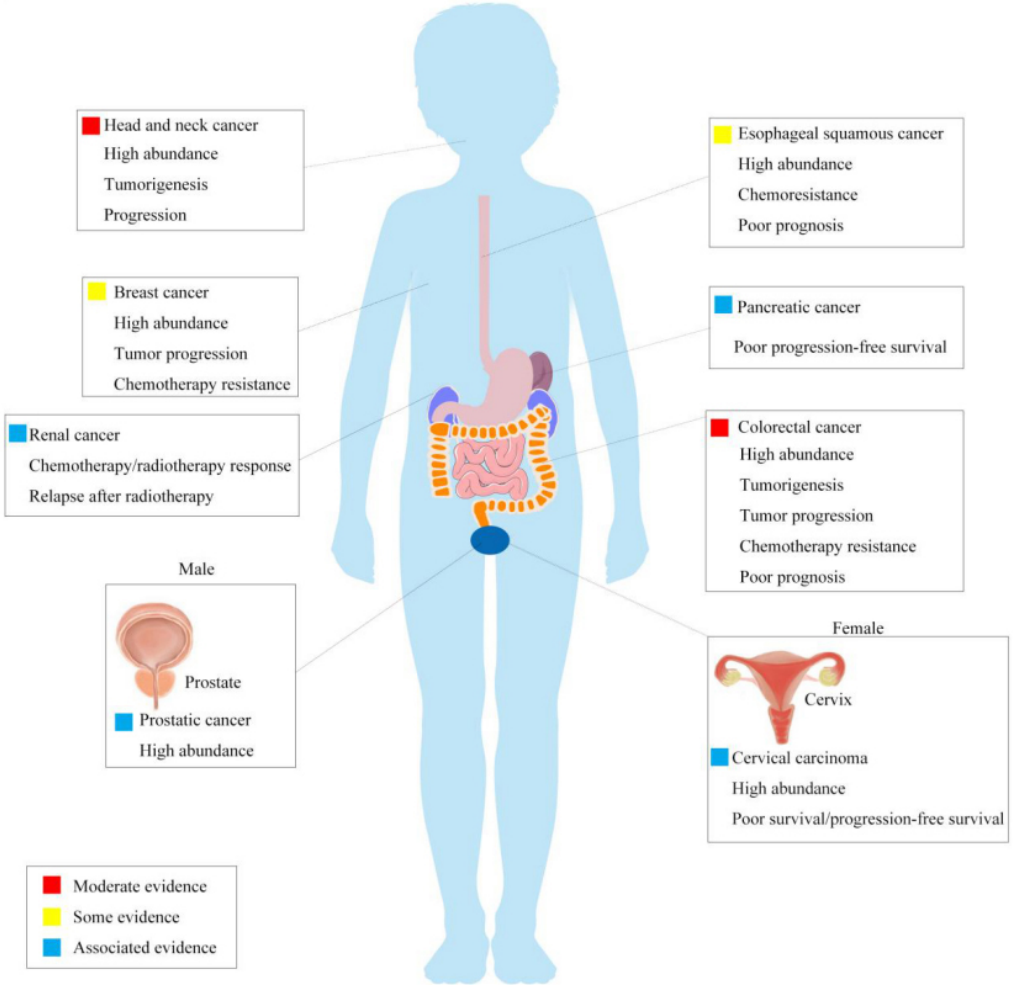

Figure 1. Different cancers associated with Fn. Fn is a Gram-negative anaerobic bacillus that exists, among others, in the human oral cavity and the gastrointestinal tract. For carcinogenesis, the high abundance of Fn is associated with head and neck cancer, esophageal cancer, pancreatic cancer, prostatic cancer, cervical carcinoma, and breast cancer. Relative events reported in each cancer are listed in the rectangles. Fn: *Fusobacterium nucleaum*.

outside the mouth? The main answer lies in two key virulence proteins expressed by Fn, FadA and Fap2, which are responsible for localization and colonization[27-30].

Mechanisms of the key virulence proteins: FadA and Fap2

FadA of Fn has previously been identified to bind host cells. FadA mainly exists in two forms: the intact pre-FadA anchored to the membrane, comprising 129 amino acids, and the secreted mature FadA (mFadA), consisting of 111 amino acids[31]. Pre-FadA and mFadA elicit internalization and form an active complex, FadAc, which leads to binding to host cell-junction molecules, cadherins[31].

Two host receptors, epithelial cadherin (E-cadherin) and vascular endothelial cadherin (VE-cadherin), are found on host epithelial and endothelial cells, respectively[32,33]. As cadherins are widely expressed in various tissues and cells, the binding of FadA to cadherins likely explains why it can colonize different sites outside oral. Further, the binding of FadAc and VE-cadherin on endothelial cells promotes VE-cadherin to transfer from cell-cell junctions to intracellular compartments, which increases the permeability of the endothelium.

After the activated FadAc binds specifically to the transmembrane domains of E-cadherin or VE-cadherin[34], the molecules form a FadA-E-cadherin-AnnexinA1 (ANXA1)-β-catenin complex, which is then internalized to induce a series host responses, such as activation of β-catenin signaling and elevated levels of oncogenic or inflammatory genes, transcription factors, and Wnt-related genes, thereby contributing to cancer initiation and promotion [Figure 2A][35].

The other Fn protein, the lectin Fap2, is an autotransporter protein. A transposon screen revealed the significance of Fap2 in mediating bacteria enrichment by binding of microbial and host cells[36]. An overexpressed D-galactose-b (1-3)-N-acetyl-D-galactosamine (Gal-GalNAc) can be recognized by fusobacterial Fap2. Then, Fap2 directly binds to Gal-GalNAc and functions as a Gal-Gal-NAc lectin to mediate Fn attachment to tumor epithelial cells[37], which subsequently inhibit immune cell cytotoxicity and activity [Figure 2B][38].

The second step: inducing host response for tumor initiation and promotion

DNA damage

DNA damage is broadly acknowledged to facilitate tumor initiation and promotion[39]. It is recognized that bacteria could directly enhance DNA methyltransferase activity in cell lines and animal models[40,41]. Fn infection is pervasive and is associated with tumor suppressor gene (TSG) promoter methylation[40], promoting high microsatellite instability (MSI-H) and a high level of CpG island methylation (CIMP-H)[42]. Kelly *et al.*[43], Mima *et al.*[44,45] and Ito *et al.*[46] discovered that a high load of Fn was positively correlated with CIMP, MSI-H, and BRAF mutation in CRC tissues by univariate analysis. Tahara *et al.*[47] also found that Fn-high CRCs were enriched in CIMP, MSI-H, hMLH1 methylation, wild-type p53, and mutant CDH7/8. Mechanistically, first, Fn might trigger TSG promoter hypermethylation by regulating the DNA methyltransferase. Interestingly, Lee *et al.*[48] also revealed that Fn of high-load patients had higher rates of transition mutation and nucleotide change of C to T (G to A) compared with Fn-low patients regardless of MSI status. Additionally, Fn-high tumors were positively correlated with a higher mutation rate of APC membrane recruitment 1 and Ataxia telangiectasia mutated genes[48]. Thus, Fn may induce promoter DNA methylation or genic mutation to drive tumorigenesis; however, the mechanism by which Fn affects these epigenetic or genetic alterations is not well understood. One potential interpretation is that Fn, involved in inflammation signals, enhances the production of reactive oxygen species (ROS) and recruits inflammatory cytokines. Although many reports suggest that ROS is associated with DNA hypermethylation, currently, there is no obvious evidence to prove that oxidative DNA damage causes genome-wide hypermethylation of promoter CpG islands and CG sites at other parts of the genome[42]. One other possible mechanism for gene alteration is the dysregulation of Checkpoint kinase 2 (Chk2), which induces cell cycle arrest and apoptosis upon DNA damage. The data imply that Chk2 is related to DNA damage and progression via Fn-induced E-cadherin/β-catenin pathway activation[49].

Once DNA damage occurs, DNA double-strand breaks (DSBs), the most serious type of DNA damage, are repaired by homologous recombination and nonhomologous end joining (NHEJ). However, a deficient repair process results in malignant transformation. Ku70, an NHEJ initiation molecule, participates in DNA damage repair signaling by initiating apoptosis programs and activating cell cycle detection points. Geng *et al.*[50] reported in 2020 that Fn could cause DNA damage and promote cell proliferation via the Ku70/p53 pathway in oral cancer cells. When suffering from an infection, Nei-like DNA glycosylase 2 (NEIL2), an oxidized base-specific DNA glycosylase, significantly relieves inflammation response and DNA damage. However, the protein level of NEIL2 is reported to be reduced in the progression of several types of cancer[51]. A recent study demonstrated a suppressed expression of NEIL2 by Fn infection, which consequently increased DSB accumulation and inflammatory responses, contributing to the initiation and progression of CRC[52].

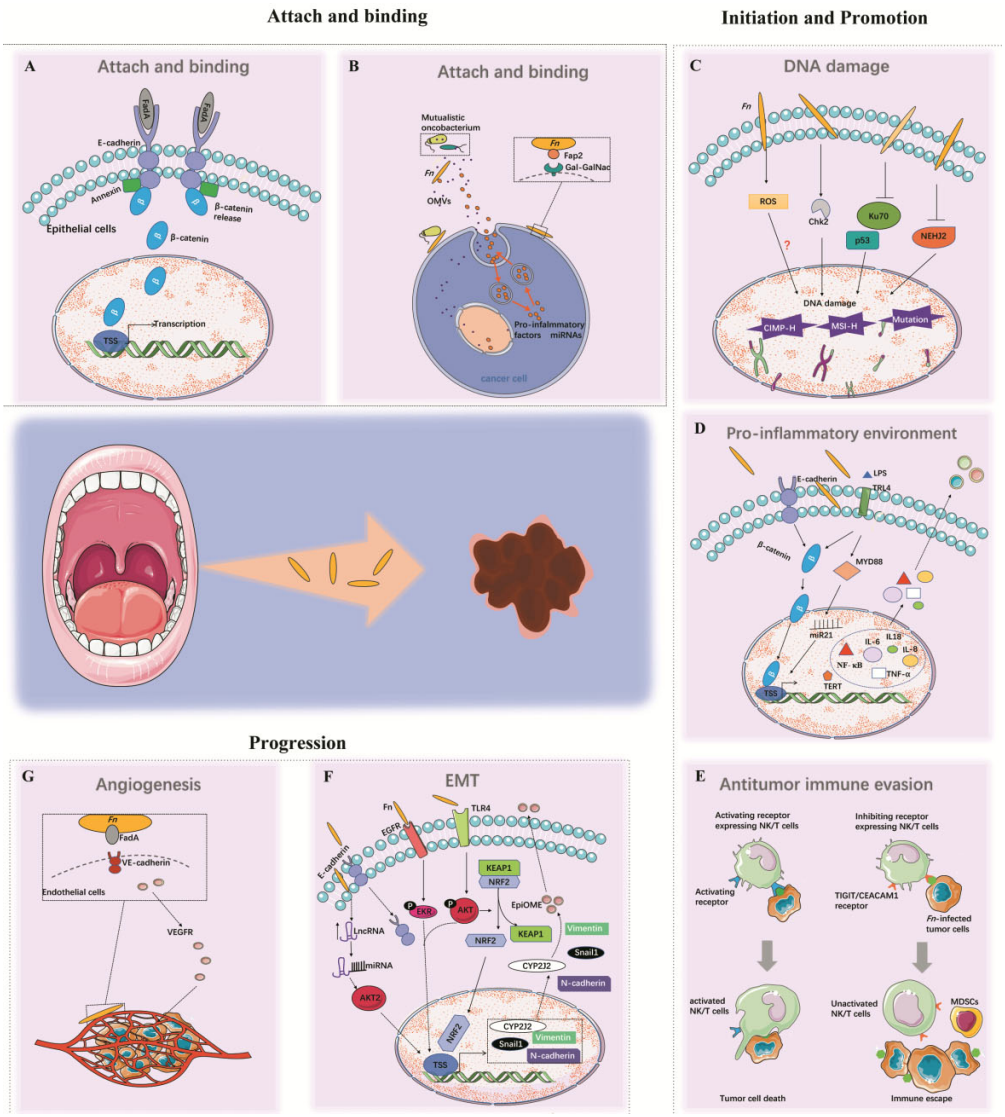

Figure 2. The mechanisms by which Fn initiates and promotes carcinogenesis and enhances disease progression. Fn plays an important role in the whole process of cancer carcinogenesis and progression, supporting the notion that Fn may have a causative role in different states of cancer rather than being a consequence of cancer development. (A,B) Mechanisms of FadA and Fap2 on host cell adherence and invasion. (C-E) Host responses for cancer initiation and promotion including DNA damage, antitumor immune evasion, and pro-inflammatory environment. (F,G) Fn-induced tumor invasion and metastasis for cancer progression. Fn: *Fusobacterium nucleaum*.

In summary, Fn infection induced promoter DNA methylation, genetic mutations, and a deficient DNA damage repair process, and hence it probably plays an essential role in tumor initiation and development [Figure 2C].

Pro-inflammatory microenvironment

Inflammation is well-recognized as a dominant force in cancer initiation. The NF-κB signaling pathway plays a vital role in activating the transcription of many inflammatory genes. Binding of FadAc to the transmembrane domains of E-cadherin induces phosphorylation and internalization of E-cadherin and accumulation of β-catenin, which consequently leads to the activation of β-catenin-regulated transcription (CRT)[32]. Alternatively, Fn also activates β-catenin signaling through its lipopolysaccharides (LPS) via a TLR4/PAK1/β-catenin S675 cascade in CRC cells[53]. Then, activated CRT increases the expression of Wnt

signaling genes (such as wnt7a, wnt7b, and wnt9a), NF-κB (such as NF-κB2), pro-inflammatory cytokines [including interleukin-6 (IL-6), IL-8 and IL-18], tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (Cox-2), transcription factors (such as the lymphoid enhancer factor), T cell factors (such as TCF1, TCF3, and TCF4), and the oncogenes myc and cyclin D1[32,54]. In addition, Fn-infected cells could enhance the expression of microRNA-21 (miR21) via activating TLR4 signaling to MYD88, which consequently activates the NF-κB pathway and elevates telomerase reverse transcriptase expression[55]. The former is correlated with the inflammatory response, while the latter confers an unlimited replicative potential to initial tumor cells[56]. Fn further promotes the release of these inflammatory cytokines, particularly IL-8, IL-10, and TNF-α, and inflammasomes to form a pro-inflammatory microenvironment that accelerates cancer progression[31,54,57]. Inflammasomes contain multiprotein complexes including the apoptosis-associated speck-like protein containing a CARD (ASC), procaspase-1, and a sensor protein, which is either a NOD-like receptor or an absent in melanoma 2 (AIM2)-like receptor[58]. In oral squamous cell carcinoma (OSCC) cells, Fn infection promotes AIM2 inflammasome expression and potentially upregulates IL-1β expression[59]. These collective findings strongly suggest that the Fn-infected tumor microenvironment is highly inflammatory [Figure 2D].

Immunosuppressive microenvironment

NK cell and T cell inhibition

Immune evasion is a fundamental hallmark of cancer. One mechanism by which Fn causes immune evasion of tumor cells is the inhibition of the cytotoxicity and activity of natural killer (NK) cells. As a part of the innate immune system, NK cells directly and indirectly kill viruses, bacteria, cancer cells, and parasites[60]. Signals from inhibiting and activating NK cell receptors determine NK cell activity. T-cell immunoglobulin and immunodominant tyrosine-based inhibitory motif domain (TIGIT), an inhibitory receptor expressed on NK cells, T cells, and tumor-infiltrating lymphocytes (TILs), inhibit NK cell and T cell activity or mediate human T cell arrest in the G1 phase of the cell cycle[61]. The direct interaction of Fap2 protein of Fn and TIGIT causes inhibition of NK cell cytotoxicity and cytotoxic T lymphocyte cell death [Figure 2E][38]. The amount of Fn is reported to be inversely associated with CD3+ T cell density in CRC tissue[62]. Another inhibitory receptor, CEACAM1, a member of the carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), is expressed on the surface of T cells and NK cells[63,64]. Previous studies reported that TILs express high levels of CEACAM1 and produce obviously less IFN-γ compared with T cells derived from para-cancer tissue, suggesting a substantial role of CEACAM1 in mediating T cell exhaustion[65]. Most recently, Fn was proved to bind to and activate CEACAM1 to inhibit T cell and NK cell activities, which may help tumors evade immune cell attack by an additive mechanism[65]. However, the functional mechanism of Fn binding to CEACAM1 is unknown and needs further investigation.

MDSC attraction

Another major immunosuppressive cell population is myeloid-derived suppressor cells (MDSCs), a group of CD11b+CD14+CD33+HLADR-immature myeloid cells that express high levels of inducible nitric oxide synthase and arginase-1 and show strong activity in depressing T cell proliferation and inducing T cell apoptosis[66]. MDSCs and their effectors are key components of the neoplasm to promote tumor progression[60,67]. Cancer-associated Fn selectively attracts MDSCs and increases the myeloid-lineage infiltrating cells including tumor-associated macrophages (TAMs), tumor-associated neutrophils, CD11b+, M2-like TAMs, conventional myeloid dendritic cells (DCs), and CD103+ regulatory DCs [Figure 2E]. These cells were shown to dampen antitumor immunity and promote tumor progression and angiogenesis[15,66,68-70].

Additional microenvironmental complexities

Fn has evolved in interaction not only with human cells and tissues but also with the oral microbiota. With its long rod shape, Fn could bind with many other microbial cells, such as *Streptococcus sanguinis* (*S.*

sanguinis)[71]. When co-cultured with *S. sanguinis*, Fn combined with *S. sanguinis*, and together they assembled into highly corncob-like structures; in this way, a single Fn can bind with up to ten *S. sanguinis* cells. This biological behavior of Fn makes it possible to mediate important biofilm-organizing behavior and interactions with host cells [Figure 2B]. In fact, Fn is usually found to be co-existent in tumors with other microorganisms, especially *Leptotrichia spp.* and *Peptostreptococcus spp.*, which mirrors how they are found to be interacting in the oral cavity[72,73]. Notably, Fn is frequently found co-occurring with *Campylobacter spp.*, another important gastrointestinal pathogen, in the same cancer tissues[73]. Continual intake of *P. gingivalis* and Fn promote tumor progression in a 4-nitroquinoline-1-oxide (4NQO)-induced mouse tongue cancer model by triggering TLR/STAT3 signaling[74]. Infection of macrophages by co-culturing with Fn and *P. gingivalis* enhances inflammasome activation more strongly than when infection with Fn alone[75]. Fn also strengthens the invasive ability of *P. gingivalis*, indicating that these bacteria may act synergistically to develop an inflammatory-permissive and tumor-promoting environment[76]. Together, these observations suggest that crosstalk between the microbiota may function as a contributor to carcinogenesis.

FINALLY: FN PROMOTE INVASION AND METASTASIS

Relatively little is known about how Fn might impact cancer progression at the later stages of carcinogenesis and metastasis, which determine poor prognosis in patients, despite the detection of Fn in CRC metastases to the liver and lymph nodes.

Epithelial-mesenchymal transition

During cancer progression, activation of the epithelial-mesenchymal transition (EMT) program is another complex hallmark, broadly facilitating local invasion and distant metastasis[77]. Expression of EMT markers is significantly associated with Fn level in CRC tissues, indicating the potential involvement of Fn in EMT-colitis-associated cancer (CAC) crosstalk during cancer progression[78]. The role of Fn in CAC progression was further verified in mouse models, as Fn apparently enhances the aggressiveness and EMT alteration of CRC cells that were treated with dextran sodium sulfate compared with untreated ones. This promoting effect of Fn was dependent on activation of the EGFR/AKT/ERK pathway[79]. Previous studies have found that Cytochrome P450 (CYP) monooxygenases, primarily Cytochrome P450 2J2 (CYP2J2), were involved in tumor progression and cancer drug resistance[80,81]. 12,13-epoxyoctadecenoic acid (12,13-EpOME), the CYP2J2-mediated metabolite product, was also reported to be associated with various human diseases[82]. A recent finding demonstrates that CYP2J2 and its oncogenic metabolite 12,13-EpOME are heavily enriched in Fn-abundance CRC patients. Further, overexpression of CYP2J2 or 12,13-EpOME dramatically promoted the invasion and migration of CRC cells and resulted in a mesenchymal phenotype. Mechanically, Fn infection activates TLR4/AKT/Keap1/NRF2 signaling to upregulate cytochrome CYP2J2 expression in CRC cells, which then increases the production of 12,13-EpOME, finally resulting in EMT[83]. Fn drives cell migration by upregulating mesenchymal markers Vimentin, N-cadherin, and snail1 in human noncancerous immortalized oral epithelial cells (OEC) and OSCC cell lines[84,85]. In a preliminary study by Fujiwar *et al.*[58], co-culture with Fn promoted the invasion of OSCC cells by upregulating EMT genes. Another study demonstrated that Fn infection could upregulate the level of lncRNA MIR4435-2HG, which specifically binds with miR-296-5p to weaken the ability of miR-296-5p to silence its target gene Akt2, subsequently activates the expression of snail1, and eventually accelerates EMT in the infected OECs [Figure 2F][86].

Extracellular product in tumor microenvironment

Different researchers using a similar *in vitro* method demonstrated that Fn infection enhances migration of human CRC-derived HCT116 cells[87]. These include inducing secretion of IL-8 and C-X-C motif chemokine ligand 1 (CXCL1), upregulating caspase activation and recruitment domain 3 (CARD3) to activate autophagy, and activating NF-κB to regulate miR-1322 or enhancer of B cell-dependent Keratin7-

antisense/Keratin7 (KRT7-AS/KRT7), which are associated with increased metastatic potential and poor prognosis[88-91].

In addition, Fn-infected CRC cells release exosomes carrying metastasis-related miR-1246/92b-3p/27a-3p and CXCL16/IL-8, particularly strongly inducing metastasis. These exosomes are then internalized to induce upregulation of β-catenin, cellular MYC proto-oncogene, cyclin D1, and the mesenchymal markers in CRC cells, implying broad cancer promoting effect of Fn exosomes and driving uninfected recipient cells toward a pro-metastatic phenotype[92].

As a Gram-negative bacterium, Fn can secret outer membrane vesicles (OMVs), containing proteins, lipoproteins, phospholipids, and LPS, which act as a delivery system for virulence factors [Figure 2B][93]. OMVs interact with host epithelial cells through surface proteins and adhesion molecules[94]. Intra-OMV proteases of Fn further degrades host E-cadherin, which facilitates bacterial invasion, inflammatory responses, and EMT.

Angiogenesis

Angiogenesis is necessary to provide nutrients and oxygen to tumor cells for further growth and tumor progression. Fn potentially activates the autocrine function of endothelial cells, resulting in a higher release of VEGF, VEGFR1, and VEGFR 2, which is favorable for proliferation and metastasis [Figure 2G][95]. In another way, Fn-attracted MDSCs reduce infiltration of T cells into the tumor and increase expression and promote secretion matrix metalloproteinase 9 (MMP-9) and MMP-13 to promote angiogenesis[79].

ROLE OF FN IN CANCER DRUG RESISTANCE

The development of drug resistance is the major cause of cancer therapy failure, affects cancer progression, and results in a poor prognosis. A growing body of evidence implies that microorganisms could modulate the host response to chemotherapeutic and immunotherapeutic drugs. Increased Fn abundance tracks with tumor stage and is associated with chemotherapy response[90,96-99]. Inhibiting the growth of Fn significantly augments the first-line chemotherapy efficiency in CRC[100]. Specific mechanisms of drug resistance may vary with different cancers and drugs. Currently, the research on Fn-mediated drug resistance mainly focuses on 5-fluorouracil (5-FU), oxaliplatin (OXA), and cisplatin (CDDP).

Autophagy pathway

The *in vitro* assay results performed by two research groups provide an insight into Fn-induced chemoresistance via the autophagy pathway in CRC and esophageal squamous cell carcinoma (ESCC) cells. They found that Fn increased the level of multiple autophagy-related genes, such as those encoding ATG7, ULK1, Beclin-1, and LC3-II. One group reported that Fn modulates the endogenous LC3 and ATG7 expression to confer chemoresistance against 5-FU, CDDP, and docetaxel in ESCC [Figure 3A][101]. Moreover, the chemoresistance induced by Fn was reversed by inhibiting autophagy through ATG7 knockdown. The results from Yu's study indicate that Fn induces CRC resistance to OXA and 5-FU. Mechanistically, Fn intervention induces a selective loss of miR-4802 and miR-18a*, leading to TLR4/MYD88-dependent autophagy activation and a CRC chemotherapeutic response [Figure 3B][102]. These studies provide insight into the role of Fn in the modulation of drug resistance through the regulation of autophagy in host cells.

Apoptosis blocking

Inhibitors of apoptosis proteins (IAPs) are characterized by the presence of baculoviral IAP repeat (BIR) domains, exerting the binding and inhibition of caspases. Baculoviral IAP repeat-containing protein 3

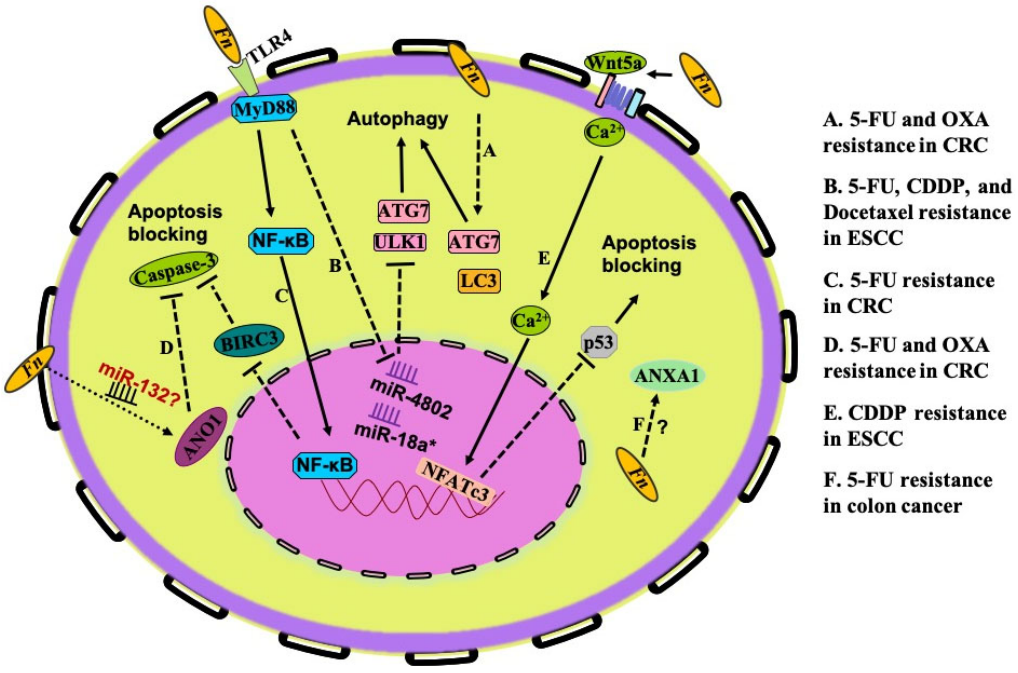

Figure 3. Specific mechanisms of drug resistance induced by Fn. The research on Fn-mediated drug resistance mainly focuses on autophagy activation (A,B) and apoptosis blockade (C-F). (A) Fn modulates endogenous LC3 and ATG7 expression to induce chemoresistance against 5-FU, CDDP, and docetaxel in ESCC. (B) Fn intervention induces a selective loss of miR-4802 and miR-18a*, leading to TLR4/MYD88-dependent autophagy activation and a CRC chemotherapeutic response to OXA and 5-FU. (C) Fn-mediated TLR4/MYD88/NF-κB pathway activation induces upregulation of BIRC3, which consequently cripples the level of cleaved caspase 3 and cleaved PARP caused by 5-FU. (D) In colon cancer cells, the apoptosis effects induced by OXA and 5-FU could be prevented by Fn-induced ANO1 upregulation. (E) Fn-induced overexpression of ANXA1 confers 5-FU resistance in colon cancer cells, but the specific mechanism needs further investigation. (F) Fn may downregulate p53 expression through the non-canonical Wnt/NFAT pathway to inhibit CDDP-induced apoptosis and migration in OSCC cells. Fn: *Fusobacterium nucleaum*; 5-FU: 5-fluorouracil; OXA: oxaliplatin; CRC: colorectal cancer; CDDP: cisplatin; ESCC: esophageal squamous cell carcinoma; BIRC3: baculoviral IAP repeat-containing protein 3; ANO1: Anoctamin-1; ANXA1: Annexin A1; OSCC: oral squamous cell carcinoma; PARP: poly ADP-ribose polymerase.

(BIRC3), a member of the IAP family, can inhibit apoptosis by directly inhibiting the caspase cascade to promote chemoresistance in malignancies[103-105]. It is reported that Fn infection protects CRCs from 5-FU-mediated apoptosis. The action mechanism is that Fn-mediated TLR4/MYD88/NF-κB pathway activation induces upregulation of BIRC3, which consequently cripples the level of cleaved caspase 3 and cleaved poly ADP-ribose polymerase (PARP) caused by 5-FU [Figure 3C][106]. Anoctamin-1 (ANO1), as one of the human chloride channel proteins, is frequently upregulated in different types of human cancers and is involved in AKT and MAPK signaling activation, which plays a critical role in cancer progression. Research data indicate that Fn promotes ANO1 expression in colon cancer cells and that the OXA- and 5-FU-induced apoptosis could be prevented by ANO1 [Figure 3D][103]. Based on the fact that ANO1 is a target of miR-132, which has a crucial role in CRC progression, and the effect of Fn on miRNAs, some authors hypothesized that Fn prevents apoptosis in CRC via the ANO1 pathway involved in modulation of the amounts of miRNA[107]. Annexin A1 (ANXA1) is a calcium-dependent phospholipid-linked protein that is involved in drug resistance, has anti-inflammatory effects, and regulates cellular differentiation, proliferation, and apoptosis. Onozawa *et al.*[108] reported that Fn-induced overexpression of ANXA1 confers 5-FU resistance in colon cancer cells, but the specific mechanism needs further investigation [Figure 3E]. The nuclear factor of activated T-cells (NFAT) is a downstream effector of the non-canonical WNT/Ca^{2+} signaling pathway that has been demonstrated to promote the migration of tumor cells and restrain apoptosis[109]. According to a study in 2021 conducted by Da *et al.*[110], Fn downregulates p53 expression to

inhibit CDDP-induced apoptosis and migration of OSCC cells. Probing into the mechanism, they found that Fn may downregulate p53 and E-cadherin through the non-canonical Wnt/NFAT pathway and induce drug resistance in Cal-27 and HSC-3 of CDDP [Figure 3F].

PREVENTION STRATEGIES

The above findings not only explain the correlation between Fn abundance and the mechanisms of tumor initiation, promotion, and progression but also raise the question of whether patients with a high abundance of Fn could benefit from an Fn-directed therapy before or concomitant with chemotherapy.

Most clinical isolates of Fn are sensitive to a number of antibiotics, including metronidazole and clindamycin and some β-lactam antibiotics. In patient-derived xenograft models of CRC with Fn enrichment, treatment with metronidazole reduced Fn load and impaired cancer cell proliferation and overall tumor growth, suggesting that Fn-abundant tumors may benefit from anti-fusobacterial therapy[72]. However, owing to the diversity of microbiota, implementing such an antibiotic intervention would be problematic in many ways. This is because antibiotics non-selectively kill both pro- and anti-tumoral bacteria. To avoid such a problem, a gut microbiota-modulatory therapy based on phage-guided biotic-abiotic hybrid nanomaterials was described by Zheng *et al.*[100]. In brief, they first isolated a phage strain from human saliva that could specifically lyse Fn. Then, they encapsulated irinotecan (IRT), a first-line drug against CRC, within dextran nanoparticles (DNPs) to form IRT-loaded DNPs (IDNPs). Finally, using a bioorthogonal reaction, they covalently linked IDNPs to azide-modified phages (A-phages) to construct a phage-guided biotic-abiotic hybrid nanosystem[100]. *In vivo* experiments were then carried out to demonstrate that A-phages accumulated in CRC tumors and that the oral administration of the nanosystem eliminated intra-tumor Fn, which inspires future treatment strategies for tumors with Fn abundance. Alternatively, an Fn-specific narrow-spectrum antibiotic might be beneficial, but due to concerns about antibiotic resistance for both broad- and narrow-spectrum antibiotics, strategies targeting virulence or interaction receptors between Fn and host cells may be more promising[15]. The Fn adhesin Fap2 and TIGIT/TLR4 may be attractive targets as they promote Fn enrichment, compromise antitumor immunity, and confer chemoresistance. An Fn-directed vaccine target at FomA (an outer membrane protein expressed by Fn that functions in bacterial co-aggregation and biofilm formation) that elicits immune response has already been tested[111]. However, data concerning the incidence of CRC after receiving the vaccine are still lacking. Further, Brennan *et al.*[15] argued that, even if the vaccination can elicit certain types of immune responses (such as human versus T cell responses), some Fn strains still escape from the immune killing effect in their intracellular phase[112]. Alternatively, T cell-inducing vaccines, similar to those vaccines targeting malaria and tuberculosis, might produce a preferable strategy for Fn. Microbial ecosystem replacement, using consortia of designed microorganisms or designed cocktails of human-derived isolates, may be another option to change the tumoral microbiota that potentially harbors Fn enrichment. This approach is clinically on trial with *Clostridium difficile* and might be tested in the future to exclude Fn.

CONCLUSION

Fn is a multidimensional bacterium that engages in interactions ranging from beneficial to detrimental in nature with other microorganisms and humans. Some researchers suggested that Fn is a passenger rather than a driver in disease states[113]. However, from the above review, we can conclude that Fn plays an important role in the whole process of cancer initiation, disease progression, and chemotherapy resistance, supporting the notion that Fn may have a causative role in different states of cancer rather than being a consequence of cancer development and chemoresistance. Nevertheless, it is still unknown how Fn transforms from a beneficial bacterium to a harmful one. Before we consider Fn-targeted treatments, we must obtain more knowledge about the basic biology of Fn. Two important challenges within this issue are:

(i) investigating how Fn strains and levels in different organs affect cancer; and (ii) understanding the mechanistic complex interactions of Fn-microbe-host within the tumor microenvironment[6]. Only by continuous investigation of the mutualistic and pathogenic characters of Fn will we reveal the divergent ways that can be used for diagnostic, preventive, and therapeutic purposes.

DECLARATIONS

Authors' contributions

Conceptualized the manuscript: Fu L
Collected the literature and wrote the manuscript: Yang K, Zhao T
Edited and made significant revisions to the manuscript: Wang X, Yang K, Fu L
All authors read and approved the final manuscript

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Opinion

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Ovarian cancer recurrence: "is the definition of platinum resistance modified by PARP inhibitors and other intervening treatments?"

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Abstract

PolyADP ribose polymerase inhibitors (PARPi) have transformed the treatment of ovarian cancer. Particularly in high-grade serous ovarian cancer (HGSOC), a disease characterized by homologous recombination deficiency (HRD), PARPi have had a rapid and profound impact on the disease course, as well as biologic and biomarker definitions of HGSOC, thereby creating a paradigm shift in the approach to treatment. In this review, we discuss the role of PARPi in the maintenance treatment of HGSOC, its effect on platinum sensitivity, and cross-resistance between platinum and PARP inhibitors.

Keywords: PARP inhibitors, olaparib, niraparib, ovarian cancer, maintenance therapy

INTRODUCTION

Ovarian cancer is a chemosensitive disease with chemosensitivity to platinum-based chemotherapy being at least in part due to defects in homologous recombination (see below). However, the majority of the patients recur after platinum-based chemotherapy, typically within 18-24 months of the treatment completion. One

of the most reliable predictors of response to subsequent chemotherapy is the duration of progression-free interval (PFI), defined as the interval between completion of the last cycle of platinum-based chemotherapy and the time of disease recurrence (progression)[1]. According to Markman's original observation, the disease that recurs within 6 months of completion of the last platinum-based chemotherapy is considered platinum resistant, whereas recurrence after a PFI of 6 months is considered platinum-sensitive [Table 1][1]. More recently, another category has been introduced - platinum refractory - for the disease that progresses during the platinum-based regimen or within 4 weeks of the last cycle [Table 1][2]. In addition, the partially platinum-sensitive disease was designated as a subgroup of the originally defined platinum-sensitive disease, and it applies to recurrences between 6-12 months from the completion of platinum-based chemotherapy [Table 1]. Initially, the definition of platinum sensitivity applied only to the first recurrence; however, subsequently, the term has been used even beyond 2nd line chemotherapy[3]. Platinum sensitivity is based on retrospective clinical observations and some clinicians consider it as a continuum. It is important to know that platinum response remains one of the most critical determinants of clinical management of patients with ovarian cancer, and it is a very important parameter in the design of clinical trials, although there has been some variability in the way the disease categories have been used in trials[4]. In general, most patients with ovarian cancer will have a platinum-sensitive disease; this group has a predictable response rate of over 60% to subsequent 2nd line chemotherapy and an expected duration of response of 9-13 months. Patients with partially platinum-sensitive disease, when treated with platinum-based chemotherapy at the time of recurrence, typically achieve a response rate of 39% and median progression-free survival (PFS) of 9.4 months[5]. In platinum-resistant disease, 16% (± 12%) of patients can be expected to demonstrate benefit, albeit in most cases with a shorter interval before disease progression[6]. Finally, primary platinum-refractory ovarian cancer is uncommon, frequently of non-high grade serous subtype, and has an unfavorable prognosis.

PARP INHIBITORS: OVERVIEW

PolyADP ribose polymerase inhibitors (PARPi) have changed the treatment landscape of ovarian cancer in a relatively short time. PARPi initially entered the clinic based on the ability to block base excision repair resulting in the accumulation of double-strand DNA breaks that were synthetically lethal with defects in homologous recombination mediated by mutations in *BRCA1/2*. High-grade serous ovarian cancer has subsequently been demonstrated to have defects in genes involved in homologous recombination DNA repair in at least 50% of cases. Based on these observations, PARPi moved quickly from the laboratory to clinic in the span of 2005 to 2009[7,8]. PARPi are approved in ovarian cancer both for treatment of recurrent disease and for maintenance of response to platin agents. Three PARPi have been approved as single-agent therapy for patients who have progressed after multiple prior lines of chemotherapy, showing remarkable activity even late in the disease course. In 2014, olaparib was approved for the 4th line treatment for patients with germline *BRCA* mutations based on the results of Study 42, a single-arm phase 2 study[9]. This was followed by the approval of rucaparib as 3rd line treatment for patients with germline and somatic *BRCA* mutations (Ariel 2 and Study 10)[10,11]. Finally, in 2019, niraparib was approved in HRD platinum-sensitive late recurrence treatment, with a remarkable response rate of 24% compared with an average 6% response rate in the late recurrent setting (Quadra trial)[12]. Subsequent studies have demonstrated activity in earlier stages of therapy and have further demonstrated combination activity with multiple different agents in ovarian cancer. While optimal activity is observed in patients with defects in homologous recombination pathway, there remains a limited activity in patients without HRD as detected by current assays. Whether this represents a failure of current assays to identify all patients with HRD or activity of PARPi outside of HRD remains to be fully elicited.

Table 1. Platinum sensitivity/resistance classification

Platinum sensitivity classification	Refractory	Resistant	Partially sensitive	Sensitive
Timing of initial progression	Chemotherapy	0-6 months→	6-12 months→	> 12 months→
Probability of 2nd line platinum response (%)	0	< 10	39	> 60

The success of the PARPi therapy studies in patients with germline and somatic *BRCA* mutated ovarian cancer opened the door to the utilization of PARPi for maintenance in the setting of recurrent platinum-sensitive ovarian cancer. In each of the subsequent 2nd line maintenance studies, PFS was extended for patients with platinum-sensitive disease, with a degree of benefit relative to genetic biomarker status [Table 2]. In SOLO2, a phase 3 study of olaparib maintenance in platinum-sensitive recurrence, there was a PFS difference of 19 *vs.* 5 months in patients with germline *BRCA* mutations receiving olaparib *vs.* placebo[13]. In Ariel 3, the PFS doubled from 5.5 to 10.8 months with a hazard ratio (HR) of 0.36 in intention to treat patients with somatic *BRCA* mutation on rucaparib maintenance[14]. The Nova trial showed remarkable efficacy of niraparib, but the degree of benefit was relative to biomarker status. Trial participants who received niraparib had a significantly longer median PFS than those in the placebo group in all three pre-specified groups: 21 *vs.* 5.5 months (HR: 0.27) for the germline *BRCA* group; 12.9 *vs.* 3.8 months (HR: 0.38) in the HRD subgroup of the non-germline *BRCA* cohort; 9.3 months *vs.* 3.9 months (HR: 0.45; 95%CI: 0.34 to 0.61) in the overall non-germline *BRCA* mutation cohort[15].

After significant success with the use of PARPi in the recurrent setting, and with 80% of patients initially platinum sensitive, PARPi were then explored as first-line maintenance in clinical trials with the hope not only for prolonged PFS, but also the extension of overall survival (OS). Of note, prior clinical trials utilizing chemotherapy with taxol and topotecan as initial maintenance therapy[16-18] showed 8 months PFS advantage, but no impact on OS[16]. Bevacizumab maintenance in the up-front setting (GOG 218) has also failed to improve OS[19]. Irreversible toxicities of taxanes and bevacizumab include neuropathy, fistula, and stroke. Therefore, prior to moving PARPi to first-line maintenance, most patients with a major response to a platinum analog were in a "watch and wait" period following completion of primary treatment.

SOLO1 changed the landscape of primary maintenance in ovarian cancer[20]. In the trial, approximately 400 patients with *BRCA* mutations (germline > somatic) were randomized to receive olaparib or a placebo. After nearly 41 months of follow-up, the treated group had a 70% lower risk of disease progression or death than the placebo group (HR: 0.30). Sensitivity analysis showed absolute longer PFS/PFI with olaparib. The median time to the first subsequent therapy or death was 51.8 months in the olaparib group *vs.* 15.1 months in the placebo group (HR: 0.30; 95%CI: 0.22 to 0.40) [Table 2]. Two other phase 3 trials in frontline maintenance were completed. Olaparib alone was compared to bevacizumab plus olaparib in the Paola-I study, which showed impressive benefit in the intent to treat a population with HR of 0.58[21]. In the Prima study within HRD population, the median duration of PFS was 22.1 months in the niraparib group and 10.9 months in the placebo group (HR: 0.40; 95%CI: 0.27 to 0.62) in the subgroup with *BRCA* mutations; in the HRD+ group with no *BRCA* mutation, median PFS was 19.6 months *vs.* 8.2 months in niraparib and placebo groups, respectively (HR: 0.50). In the subgroup of patients with homologous-recombination proficiency, the median duration of PFS was 8.1 months in the niraparib group and 5.4 months in the placebo group (HR: 0.68), leading to FDA approval of niraparib for all patients in the first-line maintenance [Table 2][22].

Table 2. PARPi maintenance trials

	Olaparib	Niraparib	Rucaparib
PARPi: first-line maintenance			
Trial design	• SOLO-1 randomized double-blind Phase 3 study • Trial size: 391 • Olaparib *vs.* placebo	• PRIMA randomized double-blind Phase 3 study • Trial size: 620 • Niraparib *vs.* placebo	
Primary endpoint (mPFS)	BRCAm+ only Not reached at 41 mo *vs.* 13.8 mo	HRD+, 19.16mo *vs.* 8.2 mo (HR: 0.50) BRCAm+ 22.1 mo *vs.* 10.9 mo (HR: 0.40) HRP 8.1 mo *vs.* 5.4 mo (HR: 0.68)	
PARPi: second-line maintenance			
Trial design	• SOLO-2 is a randomized double-blind Phase 3 trial • Trial size: 295 • Olaparib *vs.* placebo	• NOVA is a randomized double-blind Phase 3 study • Trial Size: 553 • Niraparib *vs.* placebo	• ARIEL-3 is a randomized double-blind Phase 3 study • Trial size: 564 • Rucaparib *vs.* placebo
Primary endpoint (mPFS)	• Investigator-assessed • (All) 8.4 mo. *vs.* 4.8 mo. (HR: 0.35) Study 19 Data • (BRCAm+) 19.1 mo. *vs.* 5.5 mo. (HR: 0.30)	• Blinded central review • (BRCAwt) 9.3 mo. *vs.* 3.9 mo. (HR: 0.45) • (gBRCAm+) 21.0 mo. *vs.* 5.5 mo. (HR: 0.26)	• Investigator-assessed • (All)10.8 mo. *vs.* 5.4 mo. (HR: 0.36) • (BRCAm+) 16.6 mo. *vs.* 5.4 mo. (HR: 0.23)

HRD: Homologous recombination deficiency; HR: hazard ratio.

PLATINUM AND PARP INHIBITOR RESISTANCE

As noted above, HRD contributes in part to platinum sensitivity in high-grade serous ovarian cancer. Perhaps the most cogent evidence supporting this contention is the "healing" of defects in *BRCA1/2* in patients treated with platinum analogs. This "healing" reconstitutes HR and contributes to platinum resistance. Given that resistance to PARPi is frequently due to reconstitution of HRD including "healing" of defects in *BRCA1/2*, it is reasonable to assume that PARPi treatment could contribute to resistance to platinum analogs. Furthermore, since patients can receive PARPi maintenance therapy for prolonged periods of time (> 1 year), there is a potential for PARPi to alter the response to retreatment with platinum analogs. In an alternative concept, could the prolonged period of PARPi therapy actually increase the response to platinum retreatment due to the long intervening period? At a minimum, however, the intervening treatment with PARPi requires that we redefine the concept of what period of time from prior platinum treatment would warrant retreatment with a platinum analog rather than moving to a different therapeutic alternative.

In the case of PARPi, the most important issue to address is the question of how sensitive recurrences after maintenance PARPi are to subsequent platinum-based chemotherapy due to the overlap in sensitivity and resistance mechanisms. The initial studies suggest possible cross-resistance between PARPi and platinum[23]. MITO, a retrospective study of 234 patients with *BRCA1/2*-mutations, found that patients with progression on olaparib had lower than expected response rates to subsequent platinum therapy, with a response rate of 22% in patients with a PFI > 12 months at the time of recurrence[24]. Similarly, Frenel *et al.* reported a secondary analysis of SOLO2 to show that recurrences after olaparib were less sensitive to subsequent platinum treatment compared to patients who received placebo as maintenance, with time to second progression being 14 months *vs.* 7 months in favor of the placebo group[25]. Lheureux *et al.* studied 34 patients who had progressed on a prior PARPi and were treated with olaparib and cediranib[26]. The study identified mechanisms of resistance among 19 patients: *BRCA*1/2 reversion, *BRCA*1/2 over-expression, multi-drug resistance protein overexpression, and *CCNE*1 amplification/overexpression[26].

Moreover, from ARIEL studies of rucaparib where pretreatment biopsies were required, data showed that patients with BRCA mutation reversions had a shorter PFS with rucaparib than those with no BRCA mutation reversion[27]. Other cross-resistance mechanisms to PARPi include (i) *BRCA1* alternative splicing[28]; (ii) *53BP1* loss[29]; (iii) *ABCB1* gene fusions[30]; and (iv) loss of BRCA1 methylation[27].

In patients who progress after olaparib as first-line maintenance, the time to recurrence is crucial to the definition of platinum sensitivity in the context of response to further chemotherapy. This is currently being investigated in the OREO clinical study. Although the initial results suggest that recurrences after a period of at least 24 months may respond favorably to subsequent platinum, additional analyses are needed to precisely discern the degree of platinum sensitivity and particularly the duration of response after PARPi treatment. For example, the reported median PFS in the placebo group of only 2.8 months raises the question of whether a platinum regimen has a very low activity even in responders previously treated with PARPi, or whether the high number of previous lines of therapy in the OREO trial explains the short PFS in a group of patients responding to platinum. Furthermore, one should have a clearer understanding of the degree of benefit from retreatment with a PARPi for patients with *BRCA*-associated tumors whose disease did not progress during PARPi as frontline maintenance compared to patients who were treated with PARPi with subsequent progression. A small study of 22 patients previously treated with PARPi showed that both groups experienced the benefit of retreatment with PARPi, suggesting that the development of resistance is not necessarily universal with prior exposure and progression on PARPi[31].

The complexity of biologic responses to chemotherapy after PARPi maintenance-and to some extent following bevacizumab maintenance as well-has led experts to recommend the use of treatment-free interval (TFI), as opposed to platinum sensitivity status, to more broadly assess whether intervening maintenance agents impact disease response to subsequent treatment[32]. It was proposed that TFI be defined as the period from the last disease-directed therapy, including PARPi, platinum-based, and biologic agent treatments (typically bevacizumab)[32]. The TFI concept gives us the opportunity to address unanswered questions regarding the length of maintenance treatment with PARPi as first-line maintenance. The current studies have recommended olaparib for 2 years and niraparib for 3 years in frontline maintenance. The time of recurrence and whether the recurrence occurs on treatment *vs*. after completion of prescribed maintenance is associated with the duration of platinum sensitivity. In that sense, it would be important to have a uniform established duration of the first-line maintenance treatment. Finally, there is also a need to determine whether patients who progress on PARPi after an initial response to platinum agents will benefit from retreatment with a platinum analog and to what degree compared to alternative treatment approaches.

As platinum sensitivity may be considered as a continuum, and with maintenance treatment having moved to first-line platinum responders, there is an opportunity to better understand the biological effects of PARPi on the disease response to subsequent therapies. With the response to subsequent therapy being closely related to platinum sensitivity (which is also a marker of PARPi sensitivity), this question merits further investigation via molecular analytics of serial biopsies pre- and post- first line treatment, first-line maintenance and subsequent treatment. The long-term responses in first-line PARPi maintenance treatment may indicate that a group of women will eventually be cured, which would decisively change ovarian cancer treatment and prognosis. However, patients who recur after PARPi or while on PARPi and are retreated with platinum represent the group in which we must obtain additional insight. Given the concept of retreatment with platinum analogs in patients with a prolonged PFI, a number of trials of "PARPi after PARPi" are underway. Even if "PARPi after PARPi" trials yield positive results, combination treatment with PARPi such as PARPi/Wee-i or ATRi/PARPi or PD-1/PD-L1/PARPi approaches have the potential to reverse PARPi resistance and, if toxicity allows them to be moved earlier in the treatment

spectrum, may prevent or delay the emergence of PARPi (and potentially platinum) resistance. In this manuscript, we have treated PARPi as a single modality, with this being supported by similar responses in trials across PARPi. However, different PARPi have different trapping abilities and specificity for different members of the PARP family. Further, new PARPi with greater specificity and abilities to cross the blood-brain barrier are being explored. Whether all of the PARPi will have similar effects on platinum sensitivity remains to be determined. We expect that ongoing precise and rigorously designed translational studies will, in the near future, bring more clarity to the best therapy sequence for ovarian cancer patients, and particularly identify populations of patients who are likely to benefit (or not) from platinum analogs following PARPi therapy either therapeutic or maintenance.

CONCLUSION

Today platinum remains the cornerstone of chemotherapy for ovarian cancer and PARPi are critical as a maintenance treatment. Resistance to platinum and PARPi has important clinical and prognostic significance, and the mechanisms of resistance are being rapidly investigated. A more precise understanding of the genomic markers of HRD, platinum sensitivity, and cross-resistance between PARPi and platinum will require serial biopsies (pre-, on-treatment) to be able to improve patient stratification and identify therapeutic strategies based on molecular vulnerabilities.

DECLARATIONS

Authors' contributions

Conceptualized the manuscript: Pejovic
Collected the literature and wrote the manuscript: Pejovic T
Edited and made significant revisions to the manuscript: Fitch K, Mills G
Read and approved the final manuscript: Pejovic T, Fitch K, Mills G

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Role of prostate cancer stem-like cells in the development of antiandrogen resistance

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Abstract

Androgen deprivation therapy (ADT) is the standard of care treatment for advance stage prostate cancer. Treatment with ADT develops resistance in multiple ways leading to the development of castration-resistant prostate cancer (CRPC). Present research establishes that prostate cancer stem-like cells (CSCs) play a central role in the development of treatment resistance followed by disease progression. Prostate CSCs are capable of self-renewal, differentiation, and regenerating tumor heterogeneity. The stemness properties in prostate CSCs arise due to various factors such as androgen receptor mutation and variants, epigenetic and genetic modifications leading to alteration in the tumor microenvironment, changes in ATP-binding cassette (ABC) transporters, and adaptations in molecular signaling pathways. ADT reprograms prostate tumor cellular machinery leading to the expression of various stem cell markers such as Aldehyde Dehydrogenase 1 Family Member A1 (ALDH1A1), Prominin 1 (PROM1/CD133), Indian blood group (CD44), SRY-Box Transcription Factor 2 (Sox2), POU Class 5 Homeobox 1(POU5F1/Oct4), Nanog and ABC transporters. These markers indicate enhanced self-renewal and stemness stimulating CRPC evolution, metastatic colonization, and resistance to antiandrogens. In this review, we discuss the role of ADT in prostate CSCs differentiation and acquisition of CRPC, their isolation, identification and characterization, as well as the factors and pathways contributing to CSCs expansion and therapeutic

opportunities.

Keywords: Prostate cancer, second-generation antiandrogens, cancer stem cells, castration resistance prostate cancer, androgen deprivation therapy

INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer in the United States, and approximately 50% of the men diagnosed with advance stage prostate cancer undergo androgen deprivation therapy (ADT)[1]. US Food and Drug Administration approved ADT to treat metastatic prostate cancer as a neo-adjuvant in post-radiation therapy[2]. ADT has also been accepted as the first-line treatment for prostate tumors that have extended to the lymph nodes, and biochemical recurrence followed by prostate-specific antigen (PSA) resurgence, asymptomatic metastatic and locally advanced disease[3]. Since androgen receptor (AR) is essential for the function, survival, and differentiation of prostatic tissue, ADT reduces androgens necessary to block cancer progression[4]. Accumulating data suggests that androgens are important players in the human body in maintaining physiological functions[5-6]. Androgen receptor holds a key function in prostatic epithelial cells' growth and proliferation in response to testosterone[7]. ADT comprises the use of the first-generation antiandrogens such as bicalutamide, nilutamide or flutamide that solely targets AR translocation to the nucleus and prevent downstream signaling. The second-generation antiandrogens *viz*. enzalutamide, apalutamide and darolutamide, as well as inhibitors of androgen biosynthesis such as abiraterone acetate, improve upon this mechanism[8]. Changes in the function of AR signaling result in tumor suppression to tumor promotion, where the disease eventually progresses to the emergence of castration-resistant prostate cancer (CRPC)[9]. Accumulated evidence suggests that ADT has a significant role in the management of metastatic prostate cancer; it reduces complications and enhances overall survival[2,10-11]. In a study based on Cochrane meta-analysis, the neo-adjuvant ADT with radical prostatectomy significantly improves adverse histopathologic parameters such as surgical margin or pathologic tumor stage[12]. Studies reported that prostate cancer patients undergoing radiotherapy together with ADT treatment further increases the probability of disease-free survival[12-13]. This combinatorial treatment is well accepted and highlighted in the guidelines of the American Urological Association and European Association of Urology[14-15]. However, numerous studies indicate that ADT is associated with a multitude of side effects that can impact the quality of life[16-18]. These include fatigue, loss of libido, arterial stiffness, erectile dysfunction, hot flushes, new-onset diabetes mellitus, altered body composition, osteoporosis and induced skeletal complications, and cognitive decline[16-18]. Some recent findings also demonstrated that ADT treatment might increase cardiovascular-mediated morbidity and mortality[19-20].

Prostate cancer resistance during ADT treatment is reported in *in vitro* models of recurrence and CRPC patients[21]. CRPC development is linked with genes associated with AR signaling, both at transcription and translation levels[21]. A study including multiple isogenic tumor xenograft models demonstrated increased AR expression in recurrent tumor samples compared to paired androgen-sensitive samples[22]. AR stabilization alters the rate of post-translational modifications and interaction with heat shock proteins which ultimately modulates normal cellular physiology[23]. Studies also reported that stabilization of AR is positively associated with ADT resistance, which may be linked with CRPC[4, 24]. Phosphorylation at particular sites and enrichment of growth factors reactivate AR, which further increases prostate cancer proliferation under low androgen levels[25]. Apart from AR stabilization and phosphorylation, the mutation in *AR* gene is also associated with resistance development[26]. A point mutation was reported in the lymph node of a patient with metastatic prostate cancer and causes amino acid substitution at position 878, threonine to alanine[27]. This amino acid substitution results in response to non-androgen hormones that enhance resistance to ADT treatment. Some other reported mutations are W742C, H875Y/T, F877L and

L702H in response to resistance development against enzalutamide, nilutamide, flutamide and bicalutamide[28-29]. Other factors such as different AR splice variant expression, aberrant glucocorticoids and glucocorticoid receptor expression, impairment of DNA repair pathway, miRNAs, cellular metabolism, and alterations in enzymatic and signaling pathways tightly support resistance development in response to ADT[30-31].

Emerging research implicates that cancer stem cells (CSCs) are key to the development of therapeutic resistance, and studies have also established a link to ADT relapse tumors[32]. Subsequent work has shown that ADT increases the enrichment of CSCs populations which are inherently treatment-resistant having the ability to promote CRPC[33]. The CSC hypothesis is an emerging model that describes several molecular characteristics of cancer. CSCs facilitate the development of a cellular hierarchy, maintain a CSC rich-pool, differentiate into a proliferating progeny, and assist the formation of a heterogeneous tumor[34]. As a result, there is a strong consensus that CSCs are the cells of origin in cancer and have the propensity of cancers to relapse, metastasize, and develop resistance to conventional therapies[34]. In this review, we describe the role of ADT in CSC differentiation during CRPC acquisition, their isolation, identification and characterization. Outlining the underlying mechanisms triggered by ADT and highlighting potential CSCs targets could aid in the development of future therapeutic strategies in prostate cancer patients leading to improved outcomes.

PROSTATE CANCER STEM-LIKE CELLS

Stem cell markers in prostate cancer

Normal stem cells possess the intrinsic capacity for unlimited replicative potential as well as differentiation into all lineages of mature cells required for tissue and/or organ maintenance[35]. In general, cancer cells possess cellular heterogeneity and inherent genetic instability, which makes them immortal in characteristics. CSCs are commonly defined as cells that demonstrate characteristics similar to a normal stem cell, including lack of senescence, self-renewal capacity and pluripotency[36]. This type of cancer cell possesses the ability to develop a cellular hierarchy, facilitating the maintenance of a CSC pool while differentiating into a proliferating progeny, enabling the formation and growth of a heterogeneous tumor.

The prostate gland consists of basal (undifferentiated), differentiated and neuroendocrine cells[37]. The basal cells are androgen-independent and express cytokeratin 5, cytokeratin 14, Tumor Protein P63, and Cell Surface Glycoprotein (CD44) markers[38-40]. They also express much less AR, PSA and prostatic acid phosphatase (PAP)[38-40]. Differentiated cells include glandular epithelial and secretory luminal cells, which express AR, PSA, PAP, cytokeratin 8 and cytokeratin 18[41]. The neuroendocrine cells are androgen-independent and do not secrete PSA[42]. Prostate cancer stem cells are androgen-independent cells that give rise to androgen-sensitive progenitor cells[38]. These progenitor cells in the presence of androgens differentiate into androgen-dependent cells. A number of research studies indicate that CSCs are considered as the cells of origin in cancer and have been linked to tumorigenesis, treatment resistance and cancer relapse[41].

Several research studies identified prostate CSCs genes that are important for self-renewal, pluripotency, resistance and serve as markers for identification[43]. Stem cell antigen-1, aldehyde dehydrogenases (ALDH), CD133 (PROM1), trophoblast cell surface antigen 2 and CD44 are markers to identify prostate CSCs in the basal compartment[44]. The other common markers include CD44, CD24, and CD49 Antigen-Like Family Member D (also known as Integrin Subunit Alpha 4), which have been tightly associated with prostate CSCs[45]. Another study identified a rare prostate cancer stem cell maker, KIT Proto-Oncogene, Receptor Tyrosine Kinase (KIT) in adult mouse prostatic stem cell population, which possess cancer stem cell-like

features such as differentiation and self-renewal[46]. The prostate CSCs enriched in CD133+ cells isolated from established primary human prostate cancer cell lines and in alpha(2)beta(1)-integrin subunit phenotype identified as genetically unstable with clonogenic formation and invasive potential[47]. Yu *et al.*[48] have identified high ALDH1 activity in LNCaP and PC-3 prostate cancer cells associated with CSC-like properties; in particular, $ALDH^{hi}/CD44^{+}$ cells possess a high clonogenic function and tumorigenic potential. A study conducted on prostate cancer tissue specimens indicated *Oct4*, *Sox2* and *Nanog* genes as prostate CSC markers[49]. Collins *et al.*[50] further demonstrate in the mouse xenograft model that elevated levels of ALDH support stemness in cells; indeed, $ALDH^{hi}/CD44^{+}/\alpha2\beta1^{+}$ cells are enhanced during castration and were critical in the development of antiandrogen resistance. Further, cells possessing similar phenotypes were isolated from clinical specimens and analyzed for self-renewal and spheroid formation. The outcome showed that $ALDH^{hi}/CD44^{+}/\alpha2\beta1^{+}$ cells significantly support cell proliferation and colony formation[51]. A study performed on patient biopsies samples (Gleason score range 5-6) suggested that $CD133^{+}/CD44^{+}$/ATP-binding cassette sub-family G member 2 $(ABCG2)^{+}/CD24^{-}$ cells actively participate in spheroid formation[52]. In addition, primary tumor cells containing Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit $(EZH2^{+})$/E-cadherin^{-} markers are highly associated with tumor recurrence [Table 1][53].

Molecular pathways in the generation of prostate cancer stem-like cells post ADT

AR is a key transcription factor involved in androgen-dependent prostate cancer growth. Targeting AR with the first-generation antiandrogens does not inhibit inter or intramolecular N-C interactions required for the nuclear localization[57]. At the diagnosis of metastatic CRPC, the common genomic alteration event found in AR is amplification and AR mutation[58]. These AR genomic alterations dysregulate the signaling pathway in patients and demonstrate a compensatory resistance mechanism *via* increasing AR expression in response to the potent AR inhibition by enzalutamide, which results in diminished efficacy of treatment overtime[59]. In a systemic study, exome sequencing of 150 metastatic CRPC biopsy specimens demonstrated 63% of AR mutation and amplification in comparison to 440 primary prostate cancer tissues[60]. Apart from AR mutation, AR variants such as ARV7 were also reported for resistance development and support androgen-independent growth of prostate cancer cells[61]. Prostate cancer patients who underwent ADT showed hematopoiesis from pluripotent stem cells, PI3K/AKT signaling, ERK/MAPK signaling, and Wnt/β-catenin signaling, and the role of Nanog in mammalian embryonic stem cell pluripotency signaling pathways were overrepresented [Figure 1]. This information revealed that the genomic alteration in AR either by amplification or mutation tends to increase the expression of associated stem cell markers.

With reference to hematopoiesis, pluripotent stem cells, the expression of transmembrane receptors genes, which includes *CD4*, *CD247*, *CD3E*, *CD8A*, *CSF3*, *CXCL8*, and family members of immunoglobulin heavy constant gamma proteins (IGHA1, IGHD, IGHG1, IGHG2, IGHG3, IGHM) and the expression of cytokines such as IL6 and IL10 were increased in patients undergoing ADT [Table 2].

Isolation, identification and characterization of prostate cancer stem cells

Extensive work has shown that CSCs from primary prostate tumors or established cancer cell lines can be isolated from a heterogeneous population using cell surface markers, such as CD44 and CD133, aldehyde dehydrogenase (ALDH) activity using the ALDEFLUOR assay, and Hoechst dye to identify the side population[62-63]. Isolation of prostate CSCs has been performed by several groups. The first CSC isolation was performed from patients undergoing radical prostatectomy[64]. These CSCs exhibited a significant capacity for self-renewal and the ability to regenerate the phenotypically mixed populations of non-clonogenic cells. The prostate CSCs were isolated using the $CD44/\alpha2\beta1^{high}/CD133^{+}$ phenotype and demonstrated high clonogenic and invasive capacity of basal cell origin with high levels of genetic instability[64-65]. A study by Rajasekhar *et al.*[66] observed that CSCs expressing human pluripotent stem cell marker TRA-60-1^{+} /$CD151^{+}$ /$CD166^{+}$ possess high self-renewal and differentiation ability, and were able to

Table 1. Prostate cancer stem cells markers

Pathways	Markers names	Gene symbol	Ref.
Tumor progression	KIT proto-oncogene, receptor tyrosine kinase	*CD117/c-kit*	[43,54-56]
	Prominin 1	*CD133*	
	Indian blood group	*CD44*	
	α2β1 integrin	*ITGB1*	
	Integrin Subunit Alpha 6	*α6 integrin*	
	C-X-C motif chemokine receptor 4	*CXCR4*	
	Epithelial cell adhesion molecule	*EPCAM*	
	Cytokeratin 5	*KRT5*	
	Kallikrein related peptidase 3	*KLK3/PSA*	
	Tumor-associated calcium signal transducer 2	*Trop2*	
	Activated leukocyte cell adhesion molecule	*ALCAM*	
	Aldehyde dehydrogenase 1 family member A1	*ALDH1*	
	Transglutaminase 2	*TG2*	
	Enhancer of zeste 2 polycomb repressive complex 2 subunit	*EZH2*	
Metastatic colonization and growth	KIT proto-oncogene, receptor tyrosine kinase	*CD117/c*	[43,56]
	C-X-C motif chemokine receptor	*CXCR4*	
	Epithelial cell adhesion molecule	*EPCAM*	
	E-cadherin/ cadherin 1	*CDH1*	
	Indian blood group	*CD44*	
	Enhancer of zeste 2 polycomb repressive complex 2 subunit	*EZH2*	
Recurrence and therapeutic resistance	KIT proto-oncogene, receptor tyrosine kinase	*CD117/c-kit*	[43,54-56]
	α2β1 integrin	*ITGB1*	
	Integrin subunit alpha 6	*ITGA6*	
	E-cadherin/ cadherin 1	*CDH1*	
	Epithelial cell adhesion molecule	*EPCAM*	
	C-X-C motif chemokine receptor 4	*CXCR4*	
	Enhancer of zeste 2 polycomb repressive complex 2 subunit	*EZH2*	
	Aldehyde dehydrogenase 1 family member A1	*ALDH1*	
	Transglutaminase 2	*TG2*	
	Activated leukocyte cell adhesion molecule	*CD166/ALCAM*	
	Kallikrein related peptidase 3	*KLK3/PSA*	
	Androgen receptor splice variant 7	*AR-V7*	
	ATP binding cassette subfamily G member 2 (junior blood group)	*ABCG2*	
Self-renewal capacity	Prominin 1	*CD133*	[43,55]
	Cytokeratin 5	*KRT5*	
	Kallikrein related peptidase 3	*KLK3/PSA*	
	Aldehyde dehydrogenase 1 family member A1	*ALDH1*	
	Activated leukocyte cell adhesion molecule	*CD166/ALCAM*	
	C-X-C motif chemokine receptor 4	*CXCR4*	
	Tumor-associated calcium signal transducer 2	*Trop2*	
	Integrin subunit alpha 6	*α6 integrin*	
	α2β1 integrin	*ITGB1*	
	Indian blood group	*CD44*	
Stemness gene expression	Prominin 1	*CD133*	[43,54]
	Indian blood group	*CD44*	
	E-cadherin/ cadherin 1	*CDH1*	
	Kallikrein related peptidase 3	*KLK3/PSA*	
	Aldehyde dehydrogenase 1 family member A1	*ALDH1*	
	Enhancer of zeste 2 polycomb repressive complex 2 subunit	*EZH2*	

Table 2. List of genes, location, types, along with fold changes value associated with hematopoiesis pluripotent stem cells

Gene symbol	Gene name	Fold change in ADT	Location	Type(s)
CD4	CD4 molecule	1.829	Plasma membrane	transmembrane receptor
CD247	CD247 molecule	1.437	Plasma membrane	transmembrane receptor
CD3E	CD3e molecule	1.597	Plasma membrane	transmembrane receptor
CD8A	CD8a molecule	1.469	Plasma membrane	other
CSF3	colony stimulating factor 3	5.074	Extracellular space	cytokine
CXCL8	C-X-C motif chemokine ligand 8	3.395	Extracellular space	cytokine
FCER1G	Fc fragment of IgE receptor Ig	1.815	Plasma membrane	transmembrane receptor
IGHA1	immunoglobulin heavy constant alpha 1	3.2	Extracellular space	other
IGHD	immunoglobulin heavy constant delta	2.58	Extracellular space	other
IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	2.139	Extracellular space	other
IGHG2	immunoglobulin heavy constant gamma 2 (G2m marker)	3.362	Plasma membrane	other
IGHG3	immunoglobulin heavy constant gamma 3 (G3m marker)	2.922	Extracellular space	other
IGHM	immunoglobulin heavy constant mu	2.946	Plasma membrane	transmembrane receptor
IL6	interleukin 6	6.333	Extracellular space	cytokine
IL10	interleukin 10	1.764	Extracellular space	cytokine
LIF	LIF interleukin 6 family cytokine	2.636	Extracellular space	cytokine

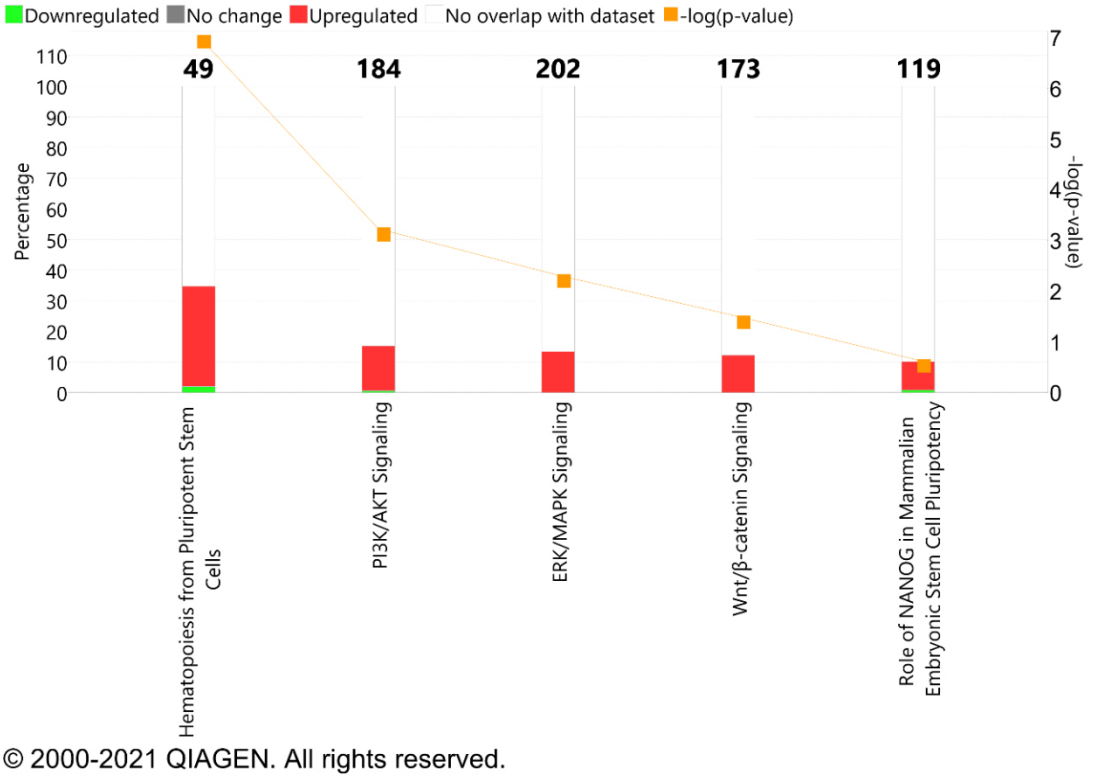

Figure 1. Canonical signaling pathway overrepresented in prostate cancer patients under ADT. These pathways play a critical role in driving cancer stem-like cell phenotype. The red bar represents the genes upregulated and green is downregulated and overlaid with IPA database. The X-axis in the graph represents the signaling pathways while the Y-axis showed -log (*P*-value) and percentage (%). ADT: Androgen deprivation therapy

reiterate tumor heterogeneity in serial *in vivo* transplantations. More recent studies have identified that a majority of prostate tumors harbor prostate-specific *TMPRSS2* gene and the *ERG* oncogene (*TMPRSS2:*

ERG) gene fusion which could be used as a stem cell marker with high specificity providing ERG-driven survival advantages[67]. Moreover, ALDH1^{high} prostate cancer cells have been shown to exhibit several CSC characteristics such as clonogenicity, migration, tumorigenicity, and propensity to form metastases *in vivo*[68]. Another method to enrich the CSC population is developing tumorspheres in cell culture with a higher *in vivo* tumor incidence rate[69].

Our group and others have demonstrated fluorescence-activated cell sorting and magnetic-activated cell sorting utilizing various human prostate cancer cell lines[70-71]. Other studies showed that CD44 and CD133 were associated with high Nanog expression in prostate carcinoma cell lines[71]. Nanog has shown to be predominantly expressed from the *NanogP8* pseudogene in a panel of prostate carcinoma cells including DU145, LNCaP, and PC-3 and primary prostate carcinoma cells. NanogP8 expression was enriched several folds in CD133$^+$ and CD133$^+$/CD44$^+$ CSCs compared to non-CSCs[71]. Human prostate cancer PC-3 cells displayed high CD44$^+$/CD133$^+$ CSC-like features including enhanced tumor sphere formation and elevated Nanog levels. Similarly, CD117$^+$/ABCG2$^+$ cells isolated from 22Rv1 prostate cancer cells overexpress the core stem cell transcription factors, Nanog, Oct3/4, and Sox2, and the CSC marker CD133[72]. A recent study from our group has demonstrated high expression of ALDH1^{high}, Oct4 and Sox2 in clinical prostate cancer specimens undergoing ADT, compared to grade-matched controls[73].

Most standard therapies for prostate cancer primarily affect cancer cells, but CSCs undergo G0/G1 phase cell cycle arrest and remain static, thus evading cell death from chemotherapeutic drugs[74]. Experimental data also suggest that CSCs are resistant to conventional chemotherapy and radiation and may be the cells responsible for disease recurrence and/or progression[75]. A study showed that CD133$^+$ had a high capacity to proliferate *in vitro* and have AR$^+$ phenotype[76]. These CD133$^+$ cells form branched spheroids structure in a 3D culture system and generate prostatic-like acini *in vivo*[76]. Hence, the drug-resistant characteristic of CSCs is useful to isolate and identify CSCs. Previous studies have shown that radiotherapy combined with hypoxic culture can also be used to enrich CSCs population[77].

Therapeutic opportunities for prostate cancer stem-like cells

Prostate cancer patients undergo treatment therapy such as radiotherapy or chemotherapy, resulting in shrinkage of tumors[78]. However, after therapy, some cells accumulate genetic/epigenetic changes that result in loss of control on self-renewal potential. These cells, referred to as prostate CSCs, reprogram the tumor environment to their benefit, supporting increased survival, self-renewal, and tumor recurrence[64]. Research showed that cellular immunotherapy has some beneficial role in the treatment of prostate cancer[79]. The T cell-based immunotherapy showed a positive response to prostate cancer patients with metastatic CRPC and increased the overall survival[79]. A research group prepared an immunogenic peptide derived from dendritic cells sensitized to CD44 and EpCAM followed by co-culture with the expanded peripheral blood lymphocyte (PBL)-derived cytokine-induced killer cell[80]. This study showed that dendritic cells- cytokine-induced killer cells exhibit remarkable cytotoxicity against prostate cancer stem-like cells-enriched prostate spheroids both *in vitro* and *in vivo*[80]. In addition to these findings, several other cellular events impart growth advantages to CSCs. In this context, various signaling pathways such as Wnt/β-catenin, hedgehog, NF-κB and Notch; ABC transporters and tumor microenvironment could be the putative target(s) for prostate CSCs[81] [Figure 2].

Wnt signaling pathway is involved in various cellular processes and is crucial for cell fate determination, cell polarity, cell migration, neural patterning and organogenesis during embryonic development. Wnt pathway is also associated with the maintenance of stem cells in a self-renewing state[82]. A study demonstrated that Wnt signaling activation is oncogenic in the prostate and supports CRPC growth *in vivo*[83]. This study also

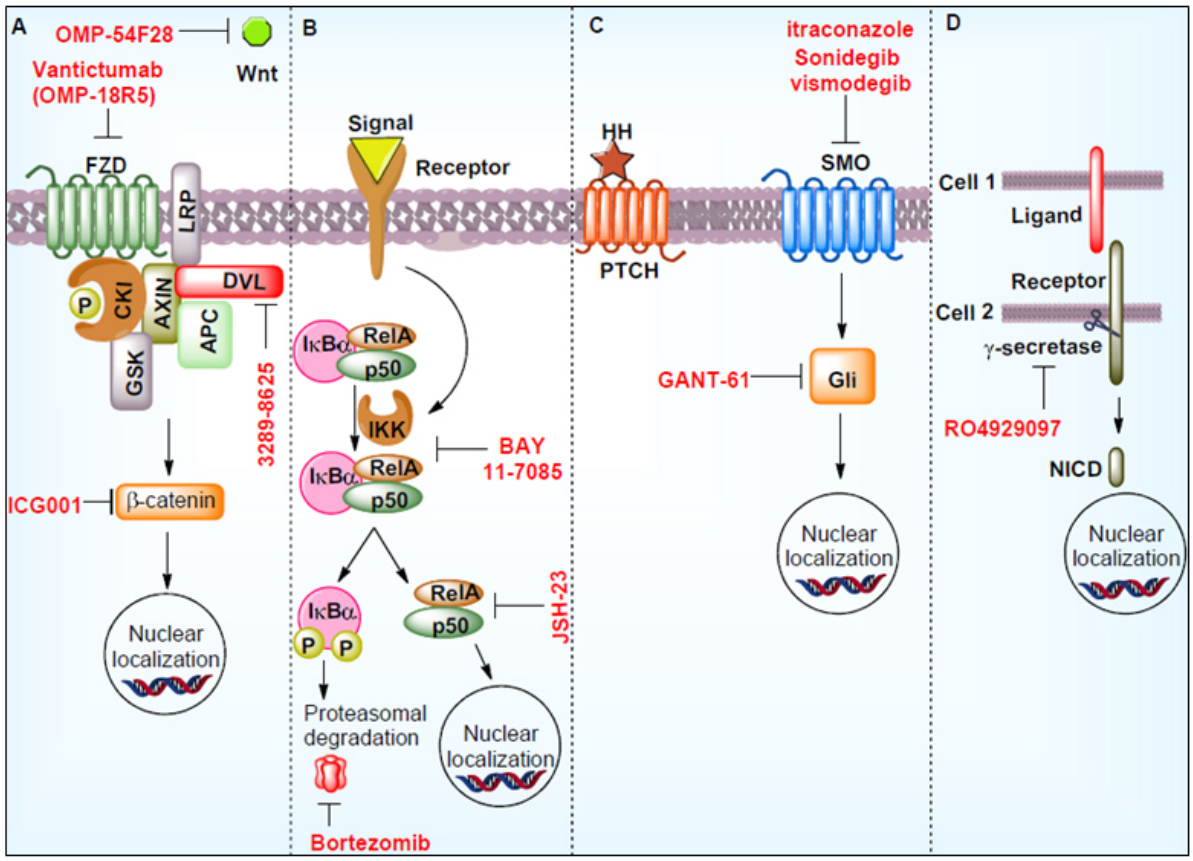

Figure 2. Signaling pathways regulating CSCs and the use of inhibitors in suppressing these pathways. These molecules thereof could be developed as potential therapeutics. The denotes in the figure are: Hedgehog signaling pathway (HH), notch intracellular domain (NICD), phosphorylation (P), smoothened (SMO), Wnt signaling pathways (Wnt), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα), RELA Proto-Oncogene (RelA), Frizzled (FZD), casein kinase I (CKI), Axin (AXIN), APC Regulator of WNT Signaling Pathway (APC), Dishevelled (Dvl), Glycogen Synthase Kinase (GSK), IkappaB Kinase (IKK).

showed that increased Wnt signaling induces neuroendocrine differentiation, epithelial-mesenchymal transition and drives stem cell-like features to prostate cancer cells. A number of small molecule inhibitors and monoclonal antibodies have been tested to inhibit the Wnt signaling pathway. Wnt signaling inhibitors such as 3289-8625, Foxy-5, and OMP-54F28 have been reported to inhibit prostate cancer cell growth[84-86]. A porcupine (palmitoylation of Wnt ligands) inhibitor LGK974 combined with docetaxel and paclitaxel also showed remarkable effectiveness on solid tumors[64]. A monoclonal antibody vantictumab (OMP-18R5) blocks canonical Wnt signaling pathways and inhibits prostate cancer progression[87].

Hedgehog signaling pathways play an important role in the development of prostate cancer. Hedgehog signaling targets genes involved in prostate CSCs survival, proliferation, and metastasis[88]. This signaling also enhanced the overexpression of ABC transporters in prostate cancer cells[88]. Hedgehog signaling inhibitor sonidegib (LDE-225) suppresses the key genes including *Oct4*, *Nanog*, *c-Myc*, and *Sox2* involved in self-renewal and stemness potential[89]. Gli transcription factor inhibitor GANT-61 inhibits PTCH1 expression and tumor growth *in vivo*[90]. Other Hedgehog signaling inhibitors such as vismodegib, itraconazole and orteronel either alone or in combination and/or surgery inhibit prostate cancer growth[64].

Upregulation of the NF-κB pathway has been observed in cancer stem cells[91]. Various studies also demonstrated that NF-κB signaling was upregulated in prostate cancer cells and associated with increased progression, chemotherapy resistance, metastasis and recurrence[92]. Several clinical trials have been performed by targeting the NF-κB signaling pathway. Clinical trial NCT01695473 used PI3K inhibitor BKM120, which acts downstream of NF-κB in high-risk localized prostate cancer patients[93]. NCT00118092 clinical trial was performed using heat shock protein 90 inhibitor 17-(allylamino)-17-demethoxygeldanamycin to treat metastatic prostate cancer patients[94]. Aspirin, a reported drug for inflammation-regulated cancer, was utilized in clinical trial NCT02757365[95]. This trial demonstrated that aspirin suppresses CRPC progression.

Notch signaling pathways are well known for their contribution to self-renewal, differentiation, resistance and stemness development[96]. Interaction of Notch receptor and ligand facilitates NICD production through gamma-secretase, which translocates to the nucleus and initiates transcription of self-renewal, stemness, and other CSC development associated genes[97]. In this context, a number of gamma-secretase inhibitory agents were identified. A clinical trial (NCT01200810) was performed in prostate cancer patients using gamma-secretase inhibitor RO4929097 with bicalutamide[98]. This trial compares PSA expression with time after surgery/radiation and combined treatment[98].

Alteration in ABC transporter genes and tumor microenvironment tightly regulates cellular physiology and transcriptomic machinery in prostate CSCs[99]. The tumor microenvironment plays a decisive role in regulating CSCs progression[100]. It also facilitates abnormal cancer signaling pathways, epithelial-mesenchymal transition, invasion, *etc*. Overexpression of ABC transporters exports therapeutic drugs outside the cells, which makes them resistant to the drug[101]. Several lines of research have been performed to target the ABC transporters. An ABC transporter efflux inhibitor verapamil inhibits prostate cancer proliferation by inhibiting the potassium ion channel[102]. Cyclosporin A, another ABC transporter inhibitor, inactivates NFATc1 (nuclear factor of activated T-cells) in biochemical recurrence and CRPC[103].

CONCLUSION

Despite thorough research for mechanisms leading to CRPC as a result of resistance to antiandrogens in the past few decades, our understanding remains limited. ADT causes complex alterations within tumors in terms of factors and pathways as well as its epigenetics and genetics affecting the tumor microenvironment. Several research studies showed that aberrant cellular signaling, generation of AR variants, and AR mutation support the development of drug resistance. The accumulative effects of these factors also contribute to the generation of prostate cancer stem-like cells. CSCs are the reservoir of cancer cells that exhibit surface markers such as ALDH, CD133 and CD44, possessing properties of self-renewal and the ability to reestablish the heterogeneous tumor cell population promoting metastatic colonization, self-renewal, and recurrence. Research showed that targeting prostate CSCs could be a better strategy for the treatment of CRPC. In this direction, various small molecule inhibitors, antibodies and other combinatorial treatments have been evaluated in various clinical trials. The outcome demonstrated that these therapies increased overall survival in prostate cancer patients. However, the lifespan increment of prostate cancer patients is still challenging for clinicians as all these drug therapies become resistant after a certain time of treatment.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and writing of the manuscript: Kushwaha PP, Verma S, Kumar S, Gupta S

Developed the figures and tables: Kushwaha PP, Verma S
Made substantial contributions to conception and design of the manuscript and performed data analysis and interpretation: Kushwaha PP, Verma S
Provided administrative, technical, and material support: Gupta S

Availability of data and materials
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Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
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Cancer Drug Resistance

Review

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An overview of resistance to Human epidermal growth factor receptor 2 (Her2) targeted therapies in breast cancer

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Abstract

Breast cancer (BC) is the second most common cause of cancer-related deaths and the most frequently diagnosed cancer in females. Among breast cancer types, HER2-positive breast cancer occurs in nearly 20% of human breast cancers and is associated with increased aggressiveness, poor prognosis, and shortened overall survival. HER2+ breast cancer is currently managed with multidisciplinary treatment strategies including surgery, radiation, chemotherapy, and targeted therapy. Drug resistance remains a continuing challenge, especially to targeted therapy utilizing monoclonal antibodies and tyrosine kinase inhibitors. This review discusses some of the recent molecular mechanisms that are involved in the development of resistance to Her2-targeted therapies including the PI3K/Akt/mTOR pathway, IGF-IR, Src, c-MET, the PP2A family, CD36, $p27^{kip1}$, and miRNAs.

Keywords: HER2+, Targeted therapy resistance, IGF-IR, Src, c-MET, PP2A, CD36, miRNA

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed cancer in females and the second most common cause of cancer-related deaths, accounting for 30% of malignancies in women[1]. In the U.S, it is estimated that approximately 40,000 women die from breast cancer each year[2]. Moreover, in 2020, 2.3 million women

were diagnosed with BC worldwide, with the number of deaths reaching 685,000. Near the end of 2020, 7.8 million women diagnosed with BC in the previous five years were alive, making it the most prevalent cancer globally[3].

Breast cancers prognosis and classification rely not only on tumor morphology but also on the expression levels of three proteins, specifically the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). Tumors that do not express any of these proteins are classified as triple-negative breast cancers, a form of the disease that is particularly difficult to treat[4].

HER2 (ERBB2) belongs to the ERBB family, which includes epidermal growth factor receptor 1 (EGFR) (ERBB1/HER1), HER3 (ERBB3), and HER4 (ERBB4)[5]. HER2 shares structural and sequence similarities with the other family members consisting of three regions: an extracellular N-terminal domain, a single transmembrane α-helix domain, and a tyrosine kinase intracellular domain[6,7].

Extracellular ligands have a conserved EGF motif bind with ERBB receptors, causing homo- and heterodimeric interactions between the ERBB receptors in various combinations[7]. HER2 has lacks a ligand binding domain and does not require a ligand for activation and may be found in an activated state via homo-dimerization or hetero-dimerization with other members of the ERBB family. Homo- or hetero-dimerization causes autophosphorylation of the tyrosine kinase domains, resulting in the subsequent activation of different signaling pathways, primarily the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) and Ras/Raf/mitogen-activated protein kinase (MAPK) pathways, promoting cell survival, proliferation, differentiation, angiogenesis, and invasion[8-11]. Although ERBB receptors are vital regulators for different cellular processes, their dysregulation, as a result of mutations, could lead to the development of cancers[7].

Amplification or overexpression of the human epidermal growth factor receptor-2 occurs in nearly 20% of human breast cancers and is associated with increased aggressiveness, poor prognosis, and short overall survival[11].

HER2+ breast cancer is currently managed with multidisciplinary treatments that include surgery, radiation, chemotherapy, and targeted therapy[12]. Targeted therapy includes monoclonal antibodies such as trastuzumab and tyrosine kinase inhibitors. Although these drugs markedly improve the prognosis of HER2+ breast cancer patients[13,14], a substantial fraction of these patients still suffer from relapse due to intrinsic or acquired resistance to the treatment, particularly in the case of trastuzumab. The majority of patients who achieve an initial response to trastuzumab-based therapy develop resistance within one year[15-17]. In fact, 70% of patients with HER2+ BC show intrinsic or acquired resistance to trastuzumab[18].

In this review, we will shed light on some of the molecular mechanisms that are involved in resistance to anti-HER2 therapies, which significantly hinder their efficacy.

SPECIFIC MECHANISMS OF RESISTANCE

Dysregulation of the PI3K/Akt/mTOR pathway

Targeted therapy resistance may occur as a result of the sustained activation of signaling pathways such as the PI3K/Akt/mTOR pathway, despite HER2 blockage, priming a drug escape mechanism[19-21].

PI3K/Akt signaling dysregulation results in mTOR pathway upregulation and increased mRNA translation leading to enhanced cellular proliferation[22,23], which is mediated by the overexpression of growth factor receptors and loss of the phosphatase and tensin homolog (PTEN) [Figure 1][24]. Breast cancer models of hyperactive PI3K/Akt/mTOR pathway have shown resistance to targeted therapy[25].

The persistent activation of this pathway may result from mutations in genes such as PIK3CA, AKT1, AKT2 amplification, and PTEN loss[26]. PTEN loss or PIK3CA mutations are common oncogenic events in HER2+ breast cancer, occurring in approximately 19% and 42% of patients, respectively[27].

PTEN, an mTOR negative regulator, is a tumor suppressor gene whose suppression leds to trastuzumab resistance and shorter survival[20,28]. Nagata *et al.*[29] provided compelling evidence supporting the role of PTEN loss in trastuzumab resistance. They showed that PI3K/Akt signaling increased via PTEN downregulation [Figure 1], which resulted in blockage of the growth arrest mediated by trastuzumab. Furthermore, they demonstrated that the absence of PTEN expression was associated with a significantly poorer response to trastuzumab-based therapy in HER2+ BC patients than in those with normal PTEN expression. Moreover, in PTEN-deficient cells, PI3K inhibitors decrease trastuzumab resistance both *in vitro* and *in vivo*[29].

PIK3CA is a gene that encodes the PI3K catalytic subunit. PIK3CA mutations acquired during disease progression are suggested to reflect increased activation of the PI3K pathway [Figure 1][19]. *In vitro* data show that PI3KCA gene mutations and HER2 gene amplification are accompanied by resistance to HER2-targeted therapy[21,30,31]. In addition, biomarker analysis from the CLEOPATRA trial showed that PIK3CA mutations were associated with worse survival outcomes in patients with advanced HER2+ breast cancer[32]. Moreover, in the EMILIA trial, PTEN loss or PIK3CA mutations were associated with shorter survival and lower overall response rates in patients receiving capecitabine and lapatinib[33].

These data support a role of the dysregulated PI3K/Akt/mTOR signaling pathway in the development of resistance to HER2 targeted agents.

c-MET

c-MET (mesenchymal-epithelial transition factor) is a tyrosine kinase receptor encoded by the proto-oncogene MET. Along with RON, c-MET belongs to the MET family, which is widely expressed in epithelial and endothelial cells[34-36]. c-MET controls a number of different cellular processes, including replication, survival, and motility[37].

c-MET becomes activated upon binding with its ligand, the hepatocyte growth factor (HGF), triggering a variety of downstream signaling pathways, including PI3K/AKT, Ras/MAPK [Figure 1], Src, signal transducer, and transcription activator[38-41].

Aberrant c-MET activation can contribute to both tumor growth and metastasis[37]. For example, c-MET was reported to be highly expressed in HER2+ BC cell lines and in 25% of HER2+ BC patients' tissues[42,43]. Poorly differentiated and invasive cell lines also showed an elevated level of c-MET[44]. Clinically, a number of trials demonstrated that c-MET hyperactivity in breast tumors is associated with a lower survival rate[43,45].

Several experimental findings suggest a role for c-MET in targeted-therapy resistance. Engelman *et al.* showed that c-MET amplification causes HER3-mediated activation of PI3K, and results in gefitinib resistance in lung cancer[46]. In addition, c-MET hyperactivity has been reported as a potential contributor to

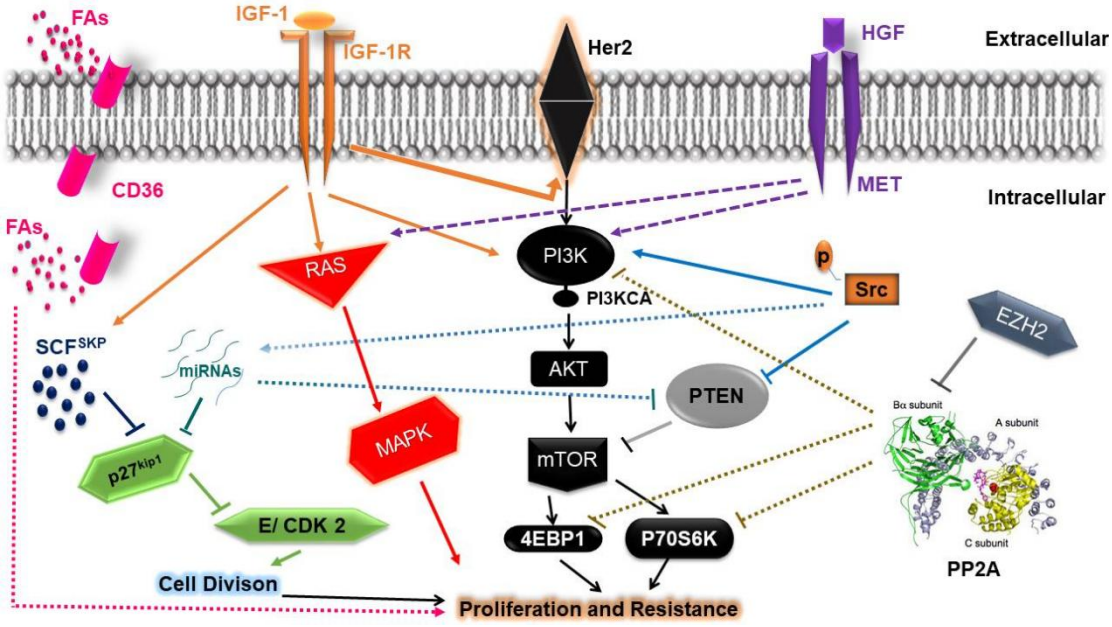

Figure 1. Signaling pathways involved in the development of resistance to Human epidermal growth factor receptor 2 (Her2)-targeted therapy. A central element of resistance appears to be PI3K/AKT/mTOR signaling, which may demonstrate persistent activation through c-MET, IGF-1, p-Src, or interference with PTEN and PP2A mediated suppression of mTOR and downstream signaling at the level of p70S6K and 4EBP1. PP2A activity could also be inhibited by EZH2-mediated slicing of the PP2A regulatory B-subunit. miRNAs and p-Src can also promote the loss of PTEN activity. Resistance could also be mediated through c-MET or IGF1 activation of the RAS/MAPK signaling pathway well as IGF-1; IGF1 can also induce Her2 receptor phosphorylation. $p27^{Kip1}$ expression is reduced via SCF^{SKP} E3 ubiquitin-mediated degradation, which can be augmented by IGF-1 or via miRNAs which are overexpressed through p-Src, causing loss of cyclin E/CDK2 control and promoting cell cycle progression. CD36 contributed to tumor growth and resistance to Her 2 targeted therapies by providing FAs as a critical energy source for tumorigenesis. PTEN: Phosphatase and tensin homolog; IGF: insulin-like growth factor; MAPK: mitogen-activated protein kinase; FAs: fatty acids.

trastuzumab resistance that may be mediated through sustained Akt activation [Figure 1][42,43]. Additionally, c-MET/HGF axis amplification was reported in a cohort of HER2+ BC patients who failed to respond to trastuzumab-based therapies[42].

Upon treatment with trastuzumab, HER2-overexpressing BC cells may upregulate c-MET, which then protects cells against trastuzumab[42]. Moreover, loss of c-MET function is reported to improve the response of these cell lines to trastuzumab[47].

In studies to demonstrate the significance of c-Met inhibition, Yue *et al.*[48] reported that miR-182 directly targets the c-MET gene in BC cells and that miR-182 downregulation is associated with trastuzumab resistance in BC cells. They utilized miR-182 to reverse the trastuzumab resistance of BC cells in part via targeting c-MET and its downstream PI3K/AKT/mTOR pathway. Their studies showed that c-MET downregulation restored sensitivity to trastuzumab *in vitro* using SKBR3 and BT474 BC cell lines, as well as in xenografted models[48].

Cell lines that have upregulated the c-MET/HGF axis have also demonstrated reduced lapatinib sensitivity, indicating that c-MET activation may decrease the effectiveness of the EGFR/HER2 inhibitors. Conversely, lapatinib or erlotinib combined with foretinib, a c-MET inhibitor, suppressed the growth of these cell lines[49,50].

Many selective c-MET inhibitors are currently under clinical development. Cabozantinib, for example, an inhibitor of c-MET and VEGFR2, is being evaluated, in combination with trastuzumab, in HER2 positive BC patients who suffer from brain metastasis[50] [Table 1].

Insulin-like growth factor 1 receptor signaling

Another pathway involved in targeted therapy resistance is mediated via the Insulin-like growth factor-I receptor (IGF-IR)[51]. IGF-IR is a tyrosine kinase receptor that plays a critical role in tumor progression. Upon ligand binding, IGF-IR initiates signaling through the Ras/MAPK and PI3K/ AKT pathways [Figure 1], resulting in cell proliferation and the inhibition of apoptosis[52].

A number of studies support a role of IGF-IR in the development of HER2+ breast cancer resistance. One study demonstrated that IGF1R interacts with and induces phosphorylation of HER2 in trastuzumab-resistant cells [Figure 1], but not in the sensitive parental cells. Moreover, the resistant cells showed more-rapid IGF1 stimulation of PI3K/Akt and Ras/MAPK pathways compared with the parental cells[53]. Furthermore, in the *in vitro* model of resistance, IGF1R signaling inhibition either by IGF1R tyrosine kinase suppression or antibody blockade restored sensitivity to trastuzumab[53].

In a study by Lu *et al.*[51], the link between trastuzumab resistance and IGF1R signaling was confirmed. These investigators demonstrated that the growth inhibition mediated by trastuzumab was lost in breast cancer cells having high HER2 and IGF1R levels. Moreover, growth arrest was restored when IGF1-mediated activation of IGF1R was inhibited by IGF-binding protein 3 (IGFBP3)[51]. IGF1 signaling was also demonstrated to elevate the expression of the $p27^{Kip1}$ ubiquitin ligase, SKP2, resulting in degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and loss of growth arrest [Figure 1][54]. $p27^{Kip1}$ belongs to the family of cyclin-dependent kinase inhibitors (CDKIs), which have inhibitory activity towards different CDKs and may function as a tumor suppressor gene by inducing cell cycle arrest[55].

In another study, cyclin-dependent kinase 2 activity was found to be increased in trastuzumab-resistant HER2+ BC cells, accompanied by a reduction in $p27^{Kip1}$ levels. The addition of exogenous $p27^{Kip1}$ increased sensitivity to trastuzumab. Thus, these data suggest that $p27^{Kip1}$ reduction is associated with trastuzumab resistance, which may be mediated by HER2 and IGF-IR heterodimerization[56,57].

$p27^{kip1}$

$p27^{kip1}$ (p27) is a member of the CDKI family[58] that functions as an inhibitor of cell cycle transition from the G1 to S phase by suppressing cyclin E/CDK 2 [Figure 1][59]. The $p27^{kip1}$ expression level serves as a prognostic marker for BC patients[60]. $p27^{kip1}$ level can be decreased at post-transcriptional levels during G1/S phase progression via ubiquitination and proteasomal degradation after its phosphorylation[61].

Several studies have investigated the role of p27 in targeted-therapy resistance, particularly to trastuzumab and lapatinib in Her2-positive breast cancer.

Trastuzumab-mediated growth arrest appears to depend, in large part, on p27 activity. Trastuzumab causes G1 cell-cycle arrest through increasing the formation of p27-CDK2 complexes[62]. Moreover, trastuzumab increases p27 half-life via decreasing its phosphorylation by cyclin E/CDK2, as well as by suppressing the subsequent ubiquitin-mediated degradation[63].

Yakes *et al.* and Le *et al.* demonstrated that the reduction of p27 levels in SKBR3 HER2+ BC cells by antisense oligonucleotides[64] or by small interfering RNA[63] blocked the growth arrest mediated by

Table 1. A summary of different targets that promote the development of Human epidermal growth factor receptor 2 (Her2)-targeted therapy resistance and drugs to potentially overcome resistance

Factors	Mechanism	Drugs	Ref.
PTEN loss	PTEN loss causes loss of PI3K/Akt/mTOR pathway control and its sustained activation	BEZ235	[29,127]
PIK3CA mutations	PIK3CA mutations increase activation of the PI3K/Akt/mTOR pathway	BEZ235	[19,127]
c-MET	Upon activation with its ligand HGF, c-MET triggers a variety of downstream signaling pathways, including PI3K/AKT, Ras/MAPK, and Src	Cabozantinib	[38-41,50]
IGF-IR	Upon ligand binding, IGF-IR initiates signaling through the Ras/MAPK and PI3K/ AKT pathways. IGF1 signaling also elevates the expression of the $p27^{Kip1}$ ubiquitin ligase, SKP2, resulting in the degradation of $p27^{Kip1}$ IGF1 signaling induces the phosphorylation of the HER2 receptor	BMS-754807	[52,53,54, 127]
$p27^{kip1}$	Alteration in its cellular localization or reduced expression is permissive for the activation of cyclin E/ CDK 2, causing cell cycle progression	MG132	[63,64,65, 56]
Src	pSrc promotes activation of the PI3K/Akt/mTOR pathway, EGFR, HER2, and HER3 receptors. Src has been found to inhibit PTEN activity and interfere with its membrane localization	Dasatinib Saracatinib	[29,78,80, 81,82]
PPP2R2B	PPP2R2B downregulation or its silencing by EZH2 causes persistent PI3K/Akt/mTOR pathway activation	EPZ-6438	[94,95,96-101,102]
CD36	The CD36-mediated pathway is activated and becomes the major source of FAs uptake rather than FASN-mediated FAs de novo biosynthesis, and provides the cell with needed energy sources, promoting tumor growth and survival	--------	[111]
MicroRNAs	MicroRNAs upregulation targets cell cycle regulators such as p57 and p27. miR-221 directly inhibits PTEN	---------	[69-71,72, 118]

PTEN: phosphatase and tensin homolog; MAPK: mitogen-activated protein kinase; PIK3CA: PIK3 catalytic subunit; HGF: hepatocyte growth factor; IGF-IR: insulin-like growth factor-I receptor; PPP2R2B: regulatory B-subunit of PP2A; pSrc: phosphorylated Src; FAs: fatty acids.

trastuzumab. Nahta *et al.* also reported that the trastuzumab-resistant SKBR3 cells showed reduced p27 levels and increased CDK2 activity[56].

Furthermore, Nahta *et al.* showed that transfection-mediated expression of p27 in the resistant cells increased trastuzumab sensitivity[56,57]. This is consistent with the findings of Kute *et al.*[65] in which p27 induction by MG132 (a proteasome inhibitor) [Table 1] restored trastuzumab sensitivity, suggesting that downregulation of p27 is likely to result from increased protein degradation. They also reported that the p27 cellular localization might be important for the response to trastuzumab, as trastuzumab-resistant BT474 HER2+ BC cells showed a loss in the nuclear expression of p27[65].

These data are consistent with earlier results in which trastuzumab exposure caused an elevation in p27 levels and nuclear localization in the sensitive cells[64].

In addition, studies by Kute *et al.*[65], Shattuck *et al.*[42], and Lu *et al.*[51,54] suggested that the IGF1R and MET signaling could contribute to p27 downregulation and the development of trastuzumab-resistance, indicating that p27 appears to be a common endpoint for various resistance pathways [42,51,53,54].

Several studies also showed that Src overexpression activated the proteolysis of p27, which may confer lapatinib-resistance in Her2 + breast cancer[66]. It was reported that Src phosphorylates p27 at Tyr74 and Tyr88 to reduce its stability and reduce p27-cyclin E-CDK2 complex formation during G1 phase[67]. Moreover, p27 phosphorylation by Src further promotes the phosphorylation of p27 by cyclin E/ CDK 2, resulting in p27 degradation by SCF^{SKP} E3 ubiquitin[68].

miR-221 upregulation has been reported in different tumors and may be involved in tumor progression by affecting expression levels of the cell cycle regulators such as p57 and p27 [Figure 1][69-71]. Interestingly,

Huynh *et al.*[72] reported that Src-activated NF-κB may result in miR-221 upregulation in lapatinib-resistant cells [Figure 1]. In addition, the findings of Huynh *et al.* indicated miR-221 involvement, and not the ubiquitination-proteasomal degradation pathway, in p27 downregulation in the lapatinib - resistant cells[72].

These data demonstrated the crosstalk of p27 with the different signaling pathways as well as its role in the development of targeted therapy resistance.

Src

The cellular proto-oncogene Src is a non-receptor tyrosine kinase that regulates varied biological processes such as cellular replication, differentiation, and survival[73,74]. Aberrant Src activation is considered to be a marked oncogenic event[75]. Src is normally found inactivated by the intramolecular binding of its phosphotyrosine (Tyr530) with the Src homology 2 domain[73]. The involvement of receptor tyrosine kinases (RTKs) with growth factors such as EGF and PDGF causes Y530 dephosphorylation and consequent Src activation[76,77]. The activated Src then autophosphorylates tyrosine 416 residue (Tyr416) in its kinase domain, enabling it to interact with a variety of targets[73].

In terms of the relationship between Src activation and the response to targeted therapy, one study which involved 57 BC patients showed that tumors with a high level of phosphorylated Src (pSrc) had a poor clinical response and more aggressive disease after trastuzumab therapy. As might have been anticipated, the overall survival was significantly lower than for patients with pSrc-low tumors[78].

Peiró *et al.*[79] demonstrated that Src activation resulted in trastuzumab resistance and poor prognosis in HER2+ breast cancer patients. Moreover, the *in vitro* inhibition of Src restored trastuzumab sensitivity in the resistant cells, with the ability to suppress tumor growth in several preclinical models of resistance. These data are consistent with findings by Zhang *et al.*[78], where expression of the activated SRC in a trastuzumab-sensitive BC cell line, BT474, caused trastuzumab resistance both *in vitro* and in xenografted mouse models. Conversely, sensitivity was restored by Src inhibition[78]. In a related finding, in a phase 2 trial of 23 patients with metastatic HER2+ BC, the combination of paclitaxel, trastuzumab, and dasatinib (Src inhibitor) showed high efficacy and success rate[80] [Table 1].

Lapatinib resistance has also been reported in cell lines showing a high level of the activated Src. Lapatinib combined with Saracatinib (Src inhibitor) significantly prolonged the xenografted-mice survival[81] [Table 1].

In efforts to understand the mechanistic relationships between the HER family, IGF-1R, PTEN, and Src, the work of Zhang *et al.*[78] showed that overexpression of EGFR or IGF-1R or PTEN loss caused Src hyperactivation that is monitored by Tyr416 phosphorylation. The activated Src then promoted trastuzumab resistance in BC cell lines via a PI3K/Akt-dependent [Figure 1] or via independent manner[78]. These cell lines also showed sensitivity to Src inhibitors[78]. Moreover, Src inhibition led to a reduction in EGFR, HER2, and HER3 activation, suggesting that the Src hyperactivity in trastuzumab-resistant cells induces a feedback loop where the active Src causes receptor activation, which, then activate Src.

Studies by Nagata *et al.*[29] and Lu *et al.*[82] also suggest a linkage between Src and PTEN, wherein PTEN can use its protein phosphatase activity to regulate Src phosphorylation. Src has been found to inhibit PTEN activity through tyrosine phosphorylation as well as by blocking the membrane localization of PTEN[29,82] [Figure 1], indicating that Src and PTEN may regulate each other to produce trastuzumab resistance.

These results suggest that Src activation is a common event during the development of targeted therapy resistance and that Src inhibition may be a novel therapeutic strategy.

The Protein Phosphatase 2A (PP2A) Family

PP2A is a serine/threonine phosphatase family that regulates a variety of cellular processes including growth, metabolism, and apoptosis[83]. PP2A family members are also well known for their role as tumor suppressors[84,85], suppressing several oncogenic pathways in carcinogenesis including Wnt, Myc, and PI3K/AKT/mTOR[86-88].

Structurally, the PP2A family is found as a heterotrimeric complex that consists of scaffolding A-subunit (PPP2R1A), regulatory B-subunit (PPP2R2B), and catalytic C-subunit (PPP2R2C)[89]. The PPP2R1A subunit often carries inactivating mutations[90,91], while the PPP2R2C and PPP2R2B subunits may be subjected to epigenetic repression or deletions[88,92,93]. These mutations suppress the PP2A tumor suppressor activity, leading to cancer development.

One critical pathway that PP2A controls is the PI3K/AKT/mTOR pathway. The ribosomal protein S6 kinase beta-1 (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) regulate two downstream pathways of AKT and mTOR that are vital for cellular proliferation and tumorigenesis[94,95]. PP2A can directly dephosphorylate p70S6K and 4EBP1 to maintain the PI3K/AKT/mTOR pathway equilibrium [Figure 1][96-101]. Consequently, PP2A may be linked to targeted-therapy sensitivity in cancer, including monoclonal antibodies and tyrosine kinase inhibitors[88,90].

In a study by Tan*et al.*[88], the silencing of PPP2R2B by DNA hypermethylation was found to be accompanied by mTOR inhibitor resistance, while Bao *et al.*[102] demonstrated that PPP2R2B downregulation is associated with poor prognosis in HER2+ BC and resistance to HER2-targeted therapy, including both lapatinib and trastuzumab. Additionally, these authors suggest that the persistent activation of both p70S6K and 4EBP1 following HER2-targeted therapy in low PPP2R2B-expressing tumor cells might result in therapy failure.

EZH2 is the catalytic subunit of the polycomb repressive complex 2, which suppresses gene expression via mediating the lysine 27 methylation of histone 3[103]. In this study by Tan *et al.*[103], PPP2R2B silencing mediated by EZH2 was shown to be required for both drug tolerance and HER2-targeted therapy resistance [Figure 1]. Moreover, HER2-targeted therapies combined with EZH2 inhibitors in anti-HER2 resistant cell lines and mouse models engrafted with trastuzumab-resistant cells showed significant tumor growth arrest in both cases. These studies suggest that the combination of EPZ-6438 (EZH2 inhibitor) with HER2-targeted therapy might prevent tumor recurrence and metastasis[102].

Taken together, these data indicate the involvement of the PP2A family in the development of HER2-targeted therapy resistance with the potential use of the PPP2R2B expression levels as a predictive marker for the response.

CD36

Fatty acids (FAs) play a vital role in various biological processes, including cellular signaling, membrane phospholipids synthesis, and energy production[104,105]. Generally, two FAs sources are available for cells to meet their energy requirements: exogenously via specialized transporters and/or endogenously via FA synthase (FASN)-mediated de novo biosynthesis[106]. In contrast to normal cells (except liver and adipose tissues), which preferentially acquire FAs from exogenous sources, more than 90% of FAs in cancer cells are

normally derived from de novo FAs synthesis[105]. Tumor cells depend on these two sources not only to sustain their proliferative capacity but also to secure a critical energy source under stress conditions[107].

In terms of exogenous FAs uptake, specific transporters are needed to facilitate FAs movement across the plasma membrane. The most commonly identified transporter is CD36[108], an 88-kDa glycosylated membrane protein that is well-known as a member of the type B scavenger receptor family. CD36 binds several ligands including anionic phospholipids, thrombospondin, and fatty acids[109].

Vazquez-Martin *et al.*[110] reported that the FASN-mediated FAs de novo biosynthesis is inhibited in response to HER2 inhibition and cancer cells undergo apoptosis, indicating crosstalk between FASN and HER2. However, a recent study found that lapatinib-resistant cells are unresponsive to (-)-C75, which is well-established to suppress FASN[111]. Furthermore, the lapatinib-resistant cells showed a significantly higher level of CD36 with an enhanced rate of FAs uptake, as well as an increase in the presence of lipid droplets compared to their sensitive counterparts[112]. CD36 expression has also been reported to be increased after anti-HER2 therapy, including lapatinib and trastuzumab, which significantly correlates with a poor prognosis in HER2+ BC patients[111]. These data suggest that there is a shift in the metabolic dependence toward CD36-mediated FAs uptake in these cells for maintaining the cellular FAs requirements[111].

CD36 knockdown via siRNA or the small molecule inhibitor, sulfosuccinimidyl oleate (SSO), re-sensitizes lapatinib-resistant cells and induces apoptotic cell death[111]. The role of CD36 in lapatinib resistance was also supported by a tumor xenograft study in mice, in which an anti-CD36 antibody markedly sensitized the resistant tumors to lapatinib. Moreover, CD36 knockdown repressed the tumor growth and extended the survival in a HER2+ BC model[111].

Illustrating the mechanistic aspects of how FAs source alteration in response to targeted therapy ultimately leads to resistance, Feng *et al.*[111] suggested that HER2 activates FASN via phosphorylation, as well as via transcriptional induction. Therefore, HER2 inhibition mediated by trastuzumab or lapatinib causes the suppression of FASN activity. The CD36-mediated pathway is then activated to become the major source of FAs uptake, promoting tumor growth and survival [Figure 1][111].

These reports indicated the possible role of CD36 in resistance to anti-Her2 targeted therapy.

MicroRNAs

MicroRNAs (miRNAs) are a class of 22-25 nucleotide RNA molecules that downregulate gene expression[113]. miRNAs also play a vital role in the post-transcriptional regulation of mRNA stability and translation efficiency through base-pairing with the complementary locations in the 3'untranslated region of the mRNA[113,114].

miRNAs have been shown to be significantly deregulated in many types of cancer, especially breast cancer, in which deregulated miRNAs are associated with breast cancer metastasis and poor prognosis in some cases, highlighting their critical roles in carcinogenesis, tumor growth, and metastasis[114-116].

The oncogenic miRNA, miR-221, is one of the few miRNAs that are persistently elevated in malignancies of different tissue including breast cancer[117], and has been suggested to accelerate cancer progression by targeting cell cycle regulators such as p57 and p27 [Figure 1][69-71].

Several studies suggest the role of miRNA in the development of targeted therapy resistance. Xingming *et al.*[118] reported that miR-221 is upregulated in breast cancer cells resistant to trastuzumab. In addition, miR-221 directly inhibits PTEN [Figure 1], causing an elevation in motility and invasiveness of HER2+ BC cells. Moreover, miR-221 suppression or the restoration of PTEN expression reversed the malignant phenotypes of HER2+ BC, indicating the critical role of miRNAs in regulating the progression of HER2+ breast cancer[118].

Huynh *et al.*[72] discovered that miR-221 upregulation by the Src/NF-κB pathway contributed to the development of lapatinib resistance by targeting p27 [Figure 1]. Furthermore, miR-221 inhibition by Src inhibitors may serve as a novel therapeutic strategy to overcome targeted-therapy resistance[72]. Interestingly, miR-16 has been reported to be involved in regulation of the NF-κB pathway[119]. In addition, miR-630 and miR-16 have been shown to play a role in HER2+ BC sensitivity to lapatinib[120-121]. The interplay among miR-16, miR-630, and miR-221 in regulating the cellular response to targeted-therapy needs to be further investigated.

CONCLUSIONS

Her2-targeted therapies are safe and effective drugs for Her2-positive breast cancer; however, de novo or acquired resistance to these agents has limited their clinical efficacy, which ultimately leads to disease relapse and tumor progression. Many molecular mechanisms are incorporated in targeted-therapy resistance including signaling through alternative RTKs[122,123], altered immune response[122], altered antibody-receptor interaction[123], Her2 TK domain mutations[124], HER3 or HER4 overexpression[125,126] and p95HER2 overexpression[50]. In this review, we have focused on recent mechanisms that play a role in resistance development and the crosstalk between different signaling pathways that contribute to disease progression and resistance. Current and future strategies for overcoming resistance include switching or combining different Her2-targeted agents and the development of small molecule inhibitors such as BEZ235 (Dual PI3K/mTOR inhibitor)[127] [Table 1], BMS-754807 (IGF1R inhibitor)[127] [Table 1], and EPZ-6438 (EZH2 inhibitor)[102] [Table 1]. These strategies show promise towards improving survival in Her2 positive BC patients.

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Authors' contributions

Made substantial contributions in terms of the organization, presentation, writing and editing of this article: Elshazly AM, Gewirtz DA

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

Review

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Molecular vulnerabilities and therapeutic resistance in hormone receptor positive and HER2 dependent breast cancer tumours

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Abstract

Over the past two decades, high sensitivity to HER2-amplified primary breast cancers has been achieved with HER2-targeted therapies. CDK4/6 inhibitors have long been identified as a potential treatment option for advanced breast cancer patients. However, acquired HER2 heterogeneity leading to resistance during the treatment has been identified as a bottleneck. This review focuses on the recent resistance mechanisms identified and potential therapeutic targets for conventional and combination endocrine therapies with CDK4/6 inhibitors by various breast cancer clinical trials and research groups in HER amplified and/or mutated breast cancer tumour. Activating *HER2* alterations, JNK pathway, hyperactivated TORC1, co-mutations in *HER2* and *HER3*, phenotypic changes of HER2, and few other advanced findings are identified as potential therapeutic targets in treating current HER2 endocrine therapy-resistant tumour. Along with the HER2-focused resistance mechanisms, we also describe how the microbiome may play a role in breast cancer therapy and its potential for new therapeutic strategies to overcome drug resistance in breast cancers.

Keywords: HER2, CDK4/6, MONALESSA-2 trial, JNK pathway, HER2 and HER3 co-mutations, microbiome, hot and cold tumour, drug resistance

INTRODUCTION

Human epidermal growth factor receptor 2 (*HER2*), a gene frequently implicated in breast cancers and a variety of other tumour types, results in a growth factor receptor, which, when upregulated, promotes tumour growth by inducing cell division. There has been a concerted effort to design cancer drugs that can inhibit the mutant HER2 protein, thereby successfully controlling the cell division and tumour growth. MONALESSA-2 trial[1] is the first report of median overall survival exceeding five years in phase 3 postmenopausal hormone receptor positive (HR+)/human epidermal growth factor receptor 2 negative (HER2-) metastatic breast cancer (MBC) when treated with cyclin-dependent kinase (CDK) CDK4/6 inhibitor with an aromatase inhibitor. Whether this is a one-off successful trial or can be replicated in other breast cancer patients will be interesting to observe. CDK4/6 inhibitors have long been identified as a potential treatment option for advanced breast cancer patients. For nearly two decades, CDK4/6 inhibitors have become the standard of care for HR+/HER2- breast cancer. A recent study analysed the association of HER2 low expression determined by immunohistochemistry score in HR positive and HER2 negative metastatic breast cancer subjects treated with CDK4/6 inhibitors. They found that the low expression of HER2 is associated with inferior progression-free survival[2]. However, not all patients achieve the expected survival outcome and become resistant to treatment regimens. Further research is needed to understand how tumour cells escape the known resistance CDK4/6 mechanisms involving E2F transcriptional activity, tumour suppressor protein RB dependent cell division arrest and senescence stage, cyclin D dependent apoptosis, and others. Hence, it is important to identify novel underlying biological mechanisms leading to resistance and cancer metastasis. Some known mechanisms leading to resistance involve the role of different coregulators (e.g., AP-1, SP-1, and AIB1), kinases (e.g., *EGFR, HER2, IGF1-R, PI3K, AKT,* and *MAPK*), and loss or modification of oestrogen receptor-α (*ESR1*) while regulating the ER signalling pathway. Since *HER2* amplification alone can act as a driver in promoting carcinogenesis, targeting both HER2 activity and interacting proteins/pathways may provide a more precise and synergistic effect on tumour regression in a combination therapy setup. The use of advanced genomic technologies and computational modelling resulted in the recent reporting of potential molecular vulnerabilities and mechanisms leading to biological breakthroughs that help advance clinical trial designs and treatment of breast cancer relatively for improved response and survival rates. Many excellent reviews have covered various breast cancer molecular vulnerabilities and resistance mechanisms[3-8]. In this review, we focus on explaining the most recent studies that proposed resistance mechanisms in *HER2* mutated breast cancers, along with the potential use of microbiome in combinatorial immunotherapies to treat breast cancer tumour for effective response.

HER2 DEPENDENT BREAST CANCER RESISTANCE MECHANISMS

Activated *HER2* alterations

Oestrogen receptor (ER), the major driver in breast cancer causation, is a known target to treat breast cancer. ER's expression not only depends on the patient's age and grade of the tumour but also on HER2 expression and loss of the *TP53* gene. CREATE-X study[9] reported that after standard neoadjuvant chemotherapy with anthracycline, taxane, or both, the addition of adjuvant capecitabine is safe and effective in improving the disease-free survival and overall survival among patients with *HER2*- breast cancer and residual invasive disease. To understand the benefits of adjuvant therapy in primary breast cancer patients, multigene tests (e.g., OncotypeDx, MammaPrint, PAM50, and others) are being performed[10-12]. These multigene tests also assist in distinguishing patients who may benefit from endocrine therapy in combination with chemotherapy. These tests stratify the tumour based on the tumour grade, risk of recurrence, and likelihood of treatment response. Endocrine therapy has been shown to be effective mostly in ER+ metastatic breast cancer patients. It is known that CDK inhibitors with endocrine therapy improve outcomes in patients with metastatic ER+ breast cancer. However, its value in early-stage patients is still unclear, and research is still limited on how varying clonal events between primary and metastatic biopsies

could result in developing resistance to endocrine therapies in combination with CDK inhibitors. With 25%-30% achieving response to endocrine therapy and others developing resistance, it becomes apparent that resistance to endocrine therapy develops during the treatment resulting in cancer metastasis. Beyond the historically known resistance mechanisms, few recent studies have shed light on how activating *HER2* mutations in ER+ metastatic breast cancer tumour had developed resistance to aromatase inhibitors, tamoxifen or fulvestrant[13]. Whole exome sequencing analysis of the primary tumour biopsies before endocrine therapy and 12 hotspot *HER2/ERBB2* mutations covering kinase, extracellular, transmembrane, and cytoplasmic domains resulted in detecting the *HER2* alterations only in the metastatic biopsies but not the primary, suggesting that these alterations were acquired during the course of therapy [Figure 1].

Clonal evolution analysis to evaluate the clonal expansion structure and dynamics also concluded similar origins of the alterations. Examination of the functional role of these alterations in T47D and MCF7 cell lines through lentiviral transduction showed strong resistance to oestrogen deprivation resistance to tamoxifen and fulvestrant. Hyperphosphorylation of both ERK and AKT under conditions of oestrogen deprivation or inhibition was also associated with the *HER2* alterations. Based on the cell viability analysis, they showed that the combined inhibition of *HER2* mutants restored sensitivity to fulvestrant. Activating *HER2* alterations are shown to offer a distinct mechanism of acquired resistance to varying ER-directed combination therapies in MBC and are postulated to be overcome by an irreversible HER2 inhibitor.

Resistance via JNK activated pathway

While the novel clonal mutations can improve understanding of the underlying resistance mechanisms and alternative therapy options, identifying the certain common characteristics or pathway alterations leading to resistance can also help offer alternative treatments. From the FELINE clinical trial[14], using exome and single-cell RNA sequencing of serially collected biopsies from ER+ breast cancer patients treated with letrozole mono endocrine therapy or in combination with different doses of CDK4/6 inhibitor ribociclib, researchers tried to understand the early-stage breast cancer evolution and develop resistance. Also, the researchers examined how disruption of CDK6, Cyclin E2 and interaction of MAPK8-10, MAPK11-14, and JNK103 could result in resistance to endocrine therapy. The study[15] identified JNK activation as offering an alternative mechanism to oestrogen-independent proliferation under combined therapy. Tumour cells with diminished endocrine signalling can bypass CDK4/6 inhibition through upregulation of the JNK pathway and showed that in cancer cells with high oestrogen signalling, potentiation of CDK4/6 activation can occur through ERBB4 and ERK upregulation and activation. In tumour without CDK6 amplification, the resistant cancer cell state was deemed to reflect a phenotypic shift from ERK to JNK MAPK signalling and reduced oestrogen signalling. When combination therapy was used in tumour without CDK6 amplification, JNK activation provided an alternative pathway to oestrogen-independent cell proliferation. Also, such showed less ERBB4 upregulation, indicating that JNK activation is a mechanism of resistance to endocrine but not CDK inhibition. Along with the activation of JNK signalling in tumour without CDK6 amplification with combination therapy, the absence of ERK signal further reflects the transition to an endocrine independent resistance state, with reduced reliance on the interaction of ESR1/ERK [Figure 2].

Both JNK and ERK pathways, which are major components of the MAPK network, showed differing activity patterns following combination therapy but not endocrine therapy alone. Along with the cell cycle, *TGF-β* and *TP53* pathway-related genes with frequent mutations, the majority of patients in the study maintained persistent polyclonal populations. Earlier reports demonstrated the stress kinase pathway via p38 and JNK to modulate ER function by phosphorylation of ER and its coregulators[16-17]. Identifying the phenotypic changes and common resistance phenotypes in the clones that survived using both exome and single-cell RNA sequencing offered an efficient method to detect resistance mechanisms. Researchers suggested that increased ERBB4 signalling, oestrogen signalling loss, responsive states, JNK pathway

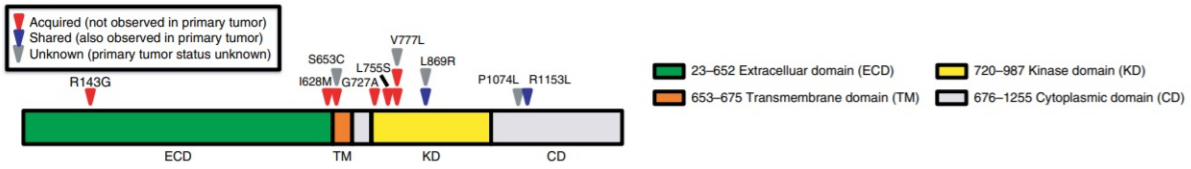

Figure 1. Acquired *HER2* alterations in patients with endocrine resistance. The location of *HER2* alterations identified by sequencing metastatic biopsies is depicted along the length of the protein. Protein domains are indicated by colour coding. Evolutionary classification for alterations: red triangles, acquired alterations; blue triangles, alterations shared with primary tumour; grey triangles, indeterminate or unknown. Figure 1 and legend used from Nayar *et al.*[13].

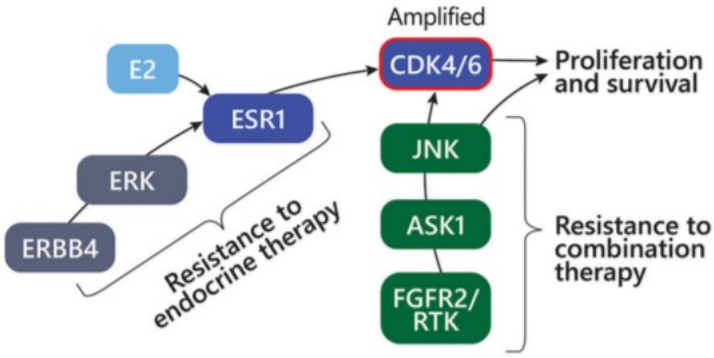

Figure 2. A schematic diagram showing resistance mechanisms driven by upregulation of ERBB4 and CDK6 amplification (red circle signifies amplification) or alternative signalling via FGFR2/RTK's and JNK signal transduction. Figure used from Griffiths *et al.*[15].

activation, and increase in basal-like tumour attribute as underlying features for developing endocrine therapy resistance and can be potential biomarkers while selecting and treating early-stage breast cancer patients[15].

Hyperactivated TORC1 driven acquired resistance

Neratinib, an irreversible pan-HER tyrosine kinase inhibitor, inhibits the growth of *HER2*-mutant tumour. It has been shown to be effective in preclinical studies in different tumour, including breast[18], colorectal, and non-small cell lung cancers with *HER2* mutations. Following that, two clinical trials MutHER[19] and SUMMIT[20] explored the efficacy of neratinib in metastatic breast cancer patients with *HER2* mutations. Initial findings reported by the SUMMIT trial indicated that neratinib plus fulvestrant combination has been shown to be clinically active in heavily pre-treated *HER2*-mutant HR+ MBC patients, including in those who had received prior fulvestrant and CDK4/6i therapy with clinical benefit rate of 47% among the trial participants[20]. Based on the varying degrees of efficacy and clinical benefits of neratinib in *HER2* mutant cancers, Sudhan *et al.*[21] postulated the role of different genomic modifiers towards the response and in developing resistance. They went on to identify clinically actionable mechanisms leading to resistance to neratinib in *HER2*-mutant cancers. Using bladder & ovarian cancer cells, they demonstrated that neratinib resistant cells showed enrichment of cell cycle promoting E2F targets, along with nuclear factor kappa B (*NF-KB*), epithelial-to-mesenchymal transition, KRAS, TORC1, inflammation, and immunological signatures. While TORC1 substrates such as p70 S6 kinase (S6K) and S6 have increased phosphorylation in drug resistance cells, EGFR, HER2, and HER3 phosphorylation were reported to be suppressed upon neratinib treatment, indicating sustained drug target inhibition. Similar activity of TORC1 was shown in neratinib-resistant breast cancer patient-derived xenografts. To confirm the role of TORC1 and to evaluate the involvement of other PI3K isoforms in neratinib's resistance, the researchers further tested the efficacy

of combining neratinib with PI3Ka (alpelisib), TORC1 (everolimus), MEK1/2 (selumetinib), AKT (MK-2206), and with the pan-PI3K inhibitor buparlisib. Pharmacological suppression testing of TORC1 resulted in identifying PI3K or MAPK inhibitors to partially suppress TORC1 activation and, hence, are less effective at reversing neratinib resistance. Further investigation revealed that both PI3K and MAPK pathways work collectively to promote TORC1 activity. Based on how RAS modulates mTOR activity through simultaneous activation of both PI3K and MAPK pathways, researchers examined RAS pathway activation status [Figure 3].

Interestingly, unlike in bladder and ovarian cancer cells, TORC1 activation in breast cancer cells was not associated with an upregulation in RAS function. This suggests that different *HER2*-mutant cancer types may develop distinct resistance mechanisms involving TORC1 signalling. The study identified hyperactivation of TORC1 as a potentially actionable mechanism driving primary and secondary resistance to neratinib in *HER2*-mutant cancers and concluded that the combination of TORC1 inhibitors with neratinib should be tested in *HER2*-mutant cancers with *de novo* or acquired mTOR pathway mutations[21].

Co-mutations in HER2 and HER3 and resistance

SUMMIT trial[22] demonstrated an effective way of probing the underlying biology among *HER2* and *HER3* mutated cancers through pharmacological HER kinase inhibition in patients. It showed an effective application of next-generation sequencing technologies in precision clinical trials while advancing our understanding of the biological impact and consequences of *HER2* and *HER3* mutations in human cancers. Although the study did not identify co-mutation of *HER2* and *HER3* in any of the study participants, concurrent aberrations in cell cycle checkpoints and activation of RTK/RAS/RAF pathway were associated with worse outcomes. While the SUMMIT trial did not report any patients with *HER2-HER3* co-mutations, another study[23] reported that co-expression of mutant HER2/HER3 enhances HER2/HER3 co-immunoprecipitation and ligand-independent activation of HER2/HER3 and PI3K/AKT, resulting in enhanced growth, invasiveness, and resistance to HER2-targeted therapies. Computational modelling and *in vitro* studies of *HER2-HER3* co-mutations showed strong binding affinity and co-mutated cells grew spikes that assisted matrix invasion, a sign of metastasis [Figure 4].

The authors suggested that patients carrying *HER2-HER3* co-mutations may not be good candidates for HER2 inhibitors alone and need a new therapeutic approach in combination with TKI and PI3Ka inhibitors to suppress the HER3 activity[23]. Although HER3 lacks an active tyrosine kinase binding domain, it has several docking sites to bind with PI3K and form heterodimers to activate PI3K pathway and improve response to PI3K inhibitors in combination with HER2 and TKI inhibitors[23].

HER2 low positive tumour and resistance mechanisms

To further understand the use of anti-HER2 agents, Denkert *et al.*[24] conducted a deep clinical and molecular analysis among 2310 patients with HER2-non-amplified primary breast cancer from four prospective, neoadjuvant trials. Based on the standardised immunohistochemistry technique, the authors reported that HER2-low-positive tumour can be identified as a new subgroup of breast cancer with distinct biology and show differences in hormone receptor status, tumour proliferation, grading, and response to neoadjuvant therapy. Significant differences in the presence of BRCA1/2 germline variants and other breast cancer predisposition genes were observed between HER2-0 and HER2-low-positive tumour. Likewise, varying frequencies of *TP53* and *PIK3CA* mutations in the two subgroups indicate different genetic and mutational backgrounds. Thus, providing enough evidence for further characterisation of the two groups to identify the mechanisms leading to sensitivity and/or resistance to treatment. A more recent study at ESMO 2021, for the first time, presented a study in which they reported a change of HER2 negative (HER2-0) to HER2 low negative tumour[25]. Among 547 primary breast tumour, they reported a 30% shift from HER2-0

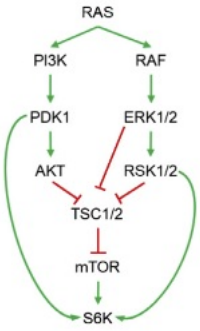

Figure 3. Schematic representation of RAS-mediated TORC1 activation by both PI3K and MAPK pathways. Figure used from Sudhan *et al.*[21].

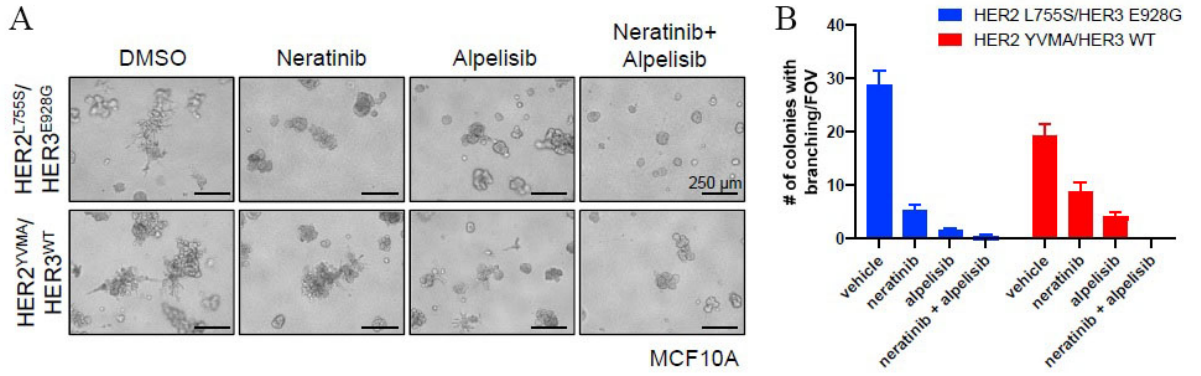

Figure 4. A: Shows MCF10A cells stably expressing the indicated genes were grown in 3D Matrigel in EGF/insulin-free medium + 1% charcoal/dextran-stripped serum (CSS) treated with vehicle [dimethyl sulfoxide (DMSO)], 20 nM neratinib, 1 mM alpelisib, or the combination. Scale bars, 250 µm. B: Shows the number of colonies with invasive branching per field of view (FOV) from (B) was quantified. Data represent the average ± SD (n = 3). Figure and legend used from Hanker *et al.*[23].

to Her2 low expression on recurrence. This switch in HER2 expression status offers more therapeutic alternatives in HER2- and also for triple-negative breast cancer patients, in which 14% tumour reportedly had the HER2 expression change. It also shows the necessity to re-test HER2 expression at relapse for improved care.

Beyond HER2 mutations and resistance mechanisms in hormone-receptor positive breast tumour

The past decade has shown the power of combining genomic sequencing and clinical information while elucidating the role of molecular vulnerabilities in targeted therapies. One such study analysed 1,918 advanced breast cancers, of which 692 tumour were previously treated with hormonal therapy. Using Memorial Sloan Kettering integrated mutation profiling of actionable cancer targets (MSK-IMPACT) platform, identified increased frequency of alterations in MAPK and oestrogen receptor transcriptional regulators and their exclusive occurrence with ESR1 mutations. The study identified *HER2* activating mutations, NF1 loss and EGFR amplification as potential therapeutic targets while treating the resistant tumour[26]. Along with understanding the role of clinically actionable mutations, it is also important to know if other variants contribute to cancer resistance. The known association of *MAP3K1*, *PIK3CA*, and *TP53* mutations with poor prognosis in luminal A and luminal B subtypes of breast cancer, using targeted sequencing, Griffith *et al.*[27] reported the significant association of frameshift nonsense (FS/NS) mutations in *NF1* and variants of unknown significance in *PIK3R1* and *DDR1* as a marker for poor prognosis and

resistance in oestrogen receptor-positive breast cancer cohort[27-28].

Transcriptomic heterogeneity and breast cancer resistance

Transcriptional heterogeneity and KDM5i therapeutic resistance

Understanding the intra- and inter- tumour heterogeneity of breast tumour has helped advance our understanding of how cancers evolve and develop resistance. The role of cancer-associated genetic alterations on transcriptomes has been very well covered in earlier reviews[29-32] and most recently in a study by pan-cancer analysis of a whole-genome consortium[33]. With growing emphasis on oncogene addiction[34] and transcriptional addiction[35], it becomes natural that more studies will focus on investigating various mechanisms leading to transcriptional addiction and resistance. Earlier, the generation of genome-wide maps of DNA-associated proteins to understand the dysregulated transcription and transcriptional heterogeneity was limited due to the number of cells that were needed[35]. However, precise measurement of transcriptional heterogeneity for effective treatment alternatives has been made possible with advances in single-cell sequencing technologies. An association of KDM5B/JARID1B, which encodes a histone H3 lysine 4 (H3K4) demethylase, with shorter disease-free survival[36] was reported in breast cancer patients treated with endocrine therapy. To understand the mechanistic contribution of the KDM5 family of histone demethylases (HDMs) to tumourigenesis and therapy resistance, Hinohara *et al.*[37] carried out functional studies using breast cancer cell lines. They hypothesised that modulating KDM5 activity will affect intra-cellular heterogeneity, subsequently resulting in therapeutic resistance. Using single-cell RNA sequencing and Mass spectrometry, they confirmed higher KDM5B expression levels in luminal subtypes compared with basal-like breast cancer cells. ER+ primary tumour with higher KDM5B expression levels was more likely to develop local and distant metastatic recurrence in tamoxifen-treated breast cancer patients. They also confirmed heterogeneous expression of both *KDM5B* and *KDM5A* genes. KDM5 inhibitor (KDM5i) treatment decreased cellular transcriptomic heterogeneity in luminal ER+ breast cancer cells[37]. Table 1 summarises the different resistance mechanisms through which breast tumour develop resistance to endocrine and combinatorial therapies. Less cellular heterogeneity could lead to more responses, thus providing evidence of an association between heterogeneity and KDM5-driven resistance.

Dormant cells, HER2 status and resistance to endocrine therapy

Another line of thought among clinicians and researchers is whether endocrine therapy works by inhibiting cell division and/or by shifting the cancer cells to dormant cells. A study using single-cell and imaging analysis by Hong *et al.*[38] investigated the contribution of clonal genetic diversity and transcriptional plasticity in both early and late phase tumour with endocrine therapy. Interestingly, they assigned therapy resistance to pre-adapted or dormant cells which had undergone transcriptomic reprogramming and copy number alterations. Earlier studies have reported that thrombospondin 1[39] and integrin[40] induce breast cancer cell proliferation and chemoresistance by the interaction between the cells and secreted molecules. Studies in mice have shown that some cancer cells with E-cadherin expression have activated HER2 and WNT-dependent migration[41] to distant organs and progesterone-induced migration of HER2+ cancer cells resulting in distant metastasis[42]. This suggests that the disseminated cancer cells remain dormant for a period of adaptation and attains positive clonal selection[43] before they proliferate[44]. The use of stage-specific pre-adapted biomarkers to characterise the pre-adapted cells could assist in detecting resistance. A recent *in vitro* study also demonstrated that activating the MET/FAK signalling axis leads to CDK4/6-independent CDK2 activation and could thus become a target to improve the response of cancers to CDK4/6-targeted therapies[45].

Tumour microenvironment, microbiome, and therapeutic resistance

The tumour microenvironment surrounding tumour cells is extremely important for their growth. The tumour microenvironment is characterized by stroma, fibroblasts, inflammatory cells, immune cells,

Table 1. Summary of breast cancer resistance mechanisms and molecular vulnerabilities identified as potential therapeutic targets

Resistance mechanism	Molecular vulnerability/potential biomarker	Study in...	Reference
Activated *HER2* alterations	Hyperphosphorylation of both ERK and AKT	Metastatic breast cancer	[13]
Activated JNK activated	Increased ERBB4 signalling, oestrogen signalling loss, and responsive states	Early breast cancers	[14]
Hyperactivated TORC1	PI3K and MAPK pathways	*HER2*-mutant tumour	[21]
Co-mutations in *HER2* and *HER3*	Activation of HER2/HER3 and PI3K/AKT	*HER2-HER3* co-mutated tumour	[23]
HER2 low positive	Varying frequencies of *TP53* and *PIK3CA* mutations	HER2 negative tumour	[24,25]
HER2 activating mutations, NF1 loss and EGFR amplification	*HER2* activating mutations, copy number aberrations in NF1 and EGFR	Advanced tumour with hormonal therapy	[26]
Variants of unknown significance driven resistance	Frameshift nonsense (FS/NS) mutations in *NF1, PIK3R1,* and *DDR1*	Oestrogen receptor-positive breast cancer	[27,28]
KDM5 driven resistance	Higher KDM5B expression	Breast cancer cell lines	[37]

vascular system, and connective tissue. As each tumour is unique, the microenvironment surrounding the tumour is also extremely diverse. The interrelationship between tumour cells and the tumour microenvironment is deeply involved in tumour growth, invasion, metastasis, and antitumour drug sensitivity and resistance. Antitumour drug resistance is acquired through diverse mechanisms, such as blocking the immune clearance of tumour cells, preventing drug absorption, and stimulating paracrine growth factors to promote tumour cell growth[46]. Changes in the tumour microenvironment can also be attributed to the microbiome[47]. Microbiome can alter the tumour microenvironment by regulating circulating inflammatory and immunocompetent cells, even in a distant tumour. In treatment, the antitumour effect of cytotoxic agents is influenced by the activation of immune cells and the microbiome[48]. Differences in the microbiome have been found to affect antitumour efficacy. A report showed that certain types of microbiome act to enhance antitumour immunity and help immune checkpoint inhibitors. Bifidobacterium induces immune-related genes in dendritic cells and enhances the antitumour effect of immune check inhibitors by inducing the activity of CD8-positive cells[49]. The *Bacteroides* species, *Bacteroides thetaiotaomicron*, and *Bacteroides fragilis* enhance the effect of anti-CTLA-4 antibodies[50]. There is growing clinical evidence that the microbiome influences the effectiveness of tumour immunotherapy. The patients who showed drug resistance or low drug efficacy had a lower microbiome diversity[51] and abundant *Ruminococcus obeum* and *Roseburia intestinalis*[52-53]. These studies suggest that if the antitumour immune activity is weak due to the insufficient microbiome, immune checkpoint inhibitors will not be sufficiently effective. The role of the microbiome in the efficacy of immunotherapy has been increasingly evaluated at the cellular level. Activation of macrophages and dendritic cells has been reported to be under the control of the microbiome[54]. The suppression of drug resistance in breast cancer could be solved by inducing the immunosuppressive "cold" tumour microenvironment, which inhibits the antitumour effect, to an immunologically active state, "hot" tumour immune microenvironment, by the microbiome. Prediction of drug efficacy and drug resistance by analysis of the intestinal microbiota and elimination of drug resistance by alteration of the intestinal microbiota may become a new therapeutic strategy in the future.

CONCLUSION

The use of advanced tumour and single-cell technologies has resulted in redefining the HER2 expression status, thus helping in identifying more accurate treatment alternatives for increased sensitivity. Reproducing similar clinical outcomes like in MONALEESA-2 trial would be more encouraging for both clinicians and patients. For researchers, such trials provide a unique opportunity to understand and elucidate specific molecular vulnerabilities and potential therapeutic targets among non-responders and

responders.

DECLARATIONS

Authors' contributions

Manuscript planning, literature, drafting, editing, revising and finalizing: Velaga R, Tanaka S, Toi M

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Cancer Drug Resistance

Review

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Transcriptional coactivator MED1 in the interface of anti-estrogen and anti-HER2 therapeutic resistance

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Abstract

Breast cancer is one of the most common cancer and leading causes of death in women in the United States and Worldwide. About 90% of breast cancers belong to ER+ or HER2+ subtypes and are driven by key breast cancer genes Estrogen Receptor and HER2, respectively. Despite the advances in anti-estrogen (endocrine) and anti-HER2 therapies for the treatment of these breast cancer subtypes, unwanted side effects, frequent recurrence and resistance to these treatments remain major clinical challenges. Recent studies have identified ER coactivator MED1 as a key mediator of ER functions and anti-estrogen treatment resistance. Interestingly, MED1 is also coamplified with HER2 and activated by the HER2 signaling cascade, and plays critical roles in HER2-mediated tumorigenesis and response to anti-HER2 treatment as well. Thus, MED1 represents a novel crosstalk point of the HER2 and ER pathways and a highly promising new therapeutic target for ER+ and HER2+ breast cancer treatment. In this review, we will discuss the recent progress on the role of this key ER/HER2 downstream effector MED1 in breast cancer therapy resistance and our development of an innovative RNA nanotechnology-based approach to target MED1 for potential future breast cancer therapy to overcome treatment resistance.

Keywords: MED1, transcription cofactor, estrogen receptor, HER2, therapy resistance, RNA nanotechnology

INTRODUCTION

Breast cancer remains one of the most common malignancies in women and is responsible for over 287,000 new diagnoses and 43,000 deaths annually in the US despite advances in treatment and detection. Based on the presence of key breast cancer biomarkers and global gene expression analyses, breast cancers can be generally subdivided into five subtypes, Estrogen Receptor (ER)+ Luminal A, Luminal B, HER2-enriched, Basal-like and the more recently identified Claudin Low subtype[1,2]. ER is the first and most prevalent breast cancer biomarker that is expressed at various levels in about 70%-75% of breast cancers, while the transmembrane receptor tyrosine kinase HER2 (Neu, ERBB2) is highly expressed, typically as a result of amplification of the genomic DNA containing *HER2* and several other genes, in around 15%-20% of human breast cancer[3-5]. ER+ and HER2+ breast cancer subtypes thus form the vast majority of breast cancer, and anti-estrogen and anti-HER2 targeted therapies have been developed and widely used in clinics for their treatment, respectively[6-8]. Although the development of these ER and HER2-targeted therapies has greatly improved the patient outcome and survival, unfortunately, unwanted side effects, frequent recurrence and resistance to these treatments remain significant clinical issues and severely hinder their effectiveness[3,9-11]. Thus, further understanding of molecular mechanisms underlying anti-estrogen and anti-HER2 treatment resistance and the development of new targeted therapies for better and safer patient care are urgently needed.

CURRENT ANTI-ESTROGEN AND ANTI-HER2 TREATMENT AND RESISTANCE MECHANISMS

Anti-estrogen (endocrine) therapies include the following types based on their mechanism of action: Selective Estrogen Receptor Modifiers (SERMs), Aromatase Inhibitors (AIs), and Selective Estrogen Receptor Degraders (SERDs)[12-14]. SERMs, such as tamoxifen and raloxifene, compete with endogenous estrogen for the hormone binding site on the estrogen receptor and preferentially recruit co-repressors to inhibit the transcription of ER target genes[8,15]. AIs, such as letrozole, anastrozole, and exemestane, function by inhibiting the aromatase enzyme essential for estrogen production in the body, thus depleting the hormone required for cancer cell growth[9,16]. SERDs, most notably fulvestrant and several others currently in clinical trials, provide some additional benefits by targeting ER for degradation upon binding to the ER[14,17]. While these drugs have greatly benefited and improved the outcome for many patients, the unwanted side effects, including increased risk for osteoporosis, arthralgia, rheumatoid arthritis, and endometrial cancer, hindered their usage and effectiveness. Furthermore, the de novo (intrinsic) or acquired resistance to these treatments is highly frequent and estimated to occur in about 50% of ER+ breast cancer patients.

It is now recognized that the resistance mechanisms for anti-estrogen therapies are complex and include genetic and epigenetic alterations of growth signaling pathways, ER itself and its coregulators and pioneer factors, intracellular heterogeneity, and interactions with the tumor microenvironment and immune system, *etc.*[18]. The expression of ER in most endocrine-resistant tumors suggests a continued but altered role of ER, commonly through crosstalk with aberrant upregulation and activation of growth factor receptor tyrosine kinases (RTKs) and intracellular cascades such as EGFR, HER2, Insulin-like Growth Factor 1 (IGF-1), FGFR, PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, CDK4/6, *etc.*[19-25]. Indeed, several therapies have recently been developed and approved to target these pathways (e.g., PI3K inhibitor Alpelisib, mTOR inhibitor Everolimus, CDK4/6 inhibitors Palbociclib, Ribociclib, and Abemaciclib) for breast cancer patients[26-30]. However, these treatments only showed limited benefit in certain patient populations and are again met with treatment discontinuation due to side effects and quick development of treatment resistance[26,31-33]. Thus, additional mechanistic and clinical investigations into the causes and molecular mechanisms of endocrine resistance and the development of new, safer and more effective treatment

strategies are still in great need despite the success and progress of anti-estrogen treatments[18].

HER2 (Neu) is a member of the EGFR family of receptor tyrosine kinases that activate and potentiate pro-growth signaling cascades. Current anti-HER2 therapies primarily include small molecules targeting the intracellular kinase domain and monoclonal antibodies targeting the extracellular domains[4,34,35]. The small molecule HER2 tyrosine kinase inhibitors include lapatinib, neratinib, and tucatinib, which have been shown to inhibit cell proliferation, amplify the response to chemotherapy and anti-estrogen therapy, and potentially help limit brain metastases[36-38]. Despite these benefits, adverse events such as diarrhea, nausea, vomiting, and hand-foot syndrome have been documented, which may lead to treatment discontinuation [36]. Monoclonal antibodies against HER2 include trastuzumab, pertuzumab, and more recently antibody-drug conjugates such as T-DM1 or T-DXd, which have trastuzumab antibodies conjugated to either the microtubule inhibitor emtansine or the topoisomerase inhibitor deruxtecan, respectively[39-41]. Unfortunately, targeted HER2 therapies come with their own side effect profile, too, including diarrhea, skin rash, and problematic levels of cardiotoxicity[42-44]. The overall response to anti-HER2 antibody therapies is limited as well, with only around one-third of HER2+ breast cancer patients ultimately benefiting from anti-HER2 antibody therapies[36]. Again, anti-HER2 therapy resistance and recurrence are also common, especially for patients with advanced and metastatic cancers, through point mutations affecting the ATP-catalytic site, alternate splicing/cleavage, and upregulation of other RTKs (e.g., HER3, IGF-1R, PI3K/Akt, and MET), *etc.*[10,45-48].

ESTROGEN RECEPTOR COACTIVATOR MED1 AND BREAST CANCER

Estrogen receptor is a member of the nuclear receptor transcription factor family that drives breast cancer through the transcription activation of its target genes. Upon binding to hormonal estrogen, ER binds to estrogen responsive elements and promotes gene transcription through stepwise and cyclic recruitment of diverse transcriptional coactivators[49]. These coactivators play a variety of roles in the transcription process, such as remodeling chromatin structure (e.g., SWI/SNF), modifying histones (p300, SRCs, PRMT1, CARM1), and recruiting additional proteins or RNAs to create a permissive environment for the assembly of transcription machinery[50-54]. However, to initiate transcription, it still needs another coactivator complex to bridge ER and transcription machinery that is known as the Mediator [Figure 1, adapted from Ref.[55]]. The Mediator complex is a complex of 25-30 subunits, and mediator subunit 1 (MED1) was found to be essential for estrogen-dependent interactions between mediator complex and estrogen receptor[56,57]. In addition to bridging ER with RNA polymerase II and transcription machinery, recent studies also support that MED1 could participate in ER-mediated gene transcription through additional mechanisms including chromosome looping, enhancer RNA transcription, liquid-liquid phase separation and formation of transcription condensates and super-enhancers, *etc.*[58-66]. Recent structural studies using crystallography, cryoelectron-microscopy and cross-linking mass spectrometry have revealed detailed Mediator interactions with transcription factors and general transcription machinery in transcription initiation and Pol II phosphorylation, *etc.*[67-70]. Future further super-resolution structural analyses of human MED1/Mediator complex with ER, other transcriptional coregulators, and even chromatin and RNAs using these and more advanced technologies will likely provide new deep insights into the regulation and diverse functions of MED1 and Mediator in these processes.

In breast cancer, it was found that MED1 is overexpressed in approximately 50% of breast cancers[71,72]. Further, MED1 is located in the genomic region containing HER2, commonly called the HER2 amplicon[73,74]. As such, MED1 coamplifies with HER2, and its expression is strongly correlated with HER2 amplification and expression in breast cancers. Interestingly, MED1 only exists in a subpopulation of mediator complex with a distinct subunit composition and enrichment of RNA polymerase II[75].

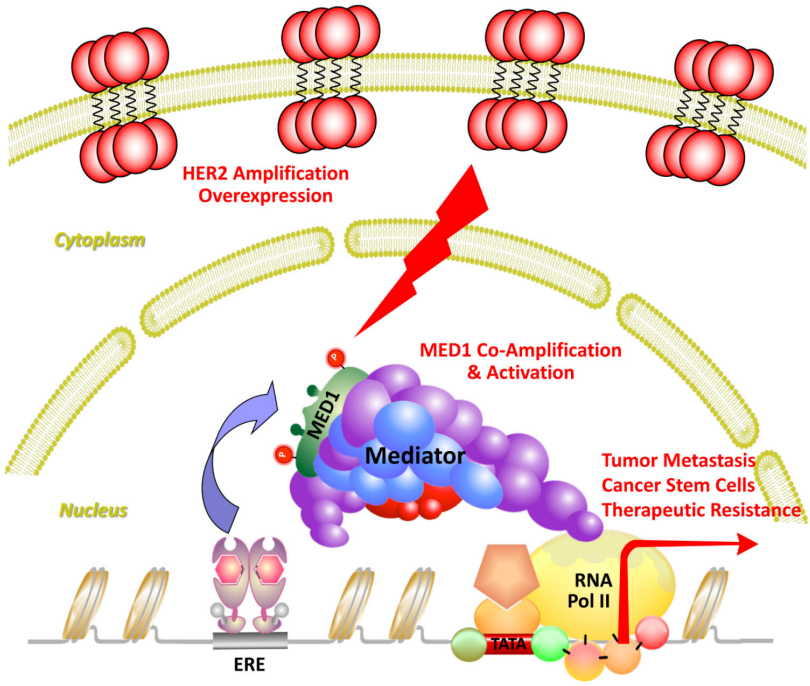

Figure 1. Transcription coactivator MED1 as a central crosstalk point of ER and HER2 pathways. Transcriptional activator MED1 directly interacts with ER to bridge it with RNA polymerase II and transcriptional machinery to initiate gene transcription and mediate ER functions in breast cancer. MED1 also coamplifies with HER2 and is activated by HER2 signaling cascades through phosphorylation to promote breast cancer growth, metastasis, stem cell expansion, and therapeutic resistance. Adapted from Yang *et al.*[55] Cell Reports.

Furthermore, the MED1/Mediator complex is selectively recruited to ER target gene promoter by estrogen induction but not to other transcription factor regulated target genes (e.g., UV activation of p53), indicating some specificity of MED1 for ER-mediated transcription function. Importantly, loss of MED1 resulted in a severe abrogation of ER target gene expression in *in vitro* transcription assays, and estrogen-dependent endogenous gene transcription and growth of breast cancer cells[56,75]. More recently, our studies further support the key *in vivo* role of MED1 in mediating selective ER functions in pubertal mammary gland development, tumor growth, metastasis, cancer stem cell formation and therapy resistance during mammary tumorigenesis as detailed in the next few sections.

TISSUE-SPECIFIC ROLE OF MED1 AND ITS LXXLL MOTIFS *IN VIVO*

Estrogen receptor interacts with a variety of transcription cofactors including MED1, SRC1, and SRC3 *etc.*, via a domain on these proteins called the LxxLL motifs or NR-boxes[76-78]. MED1 contains two closely associated LxxLL motifs, which, when mutated to LxxAA, prevent MED1 from binding to ER in a ligand-dependent manner[79]. To better understand the physiological role the MED1/ER plays, a knock-in mutant mouse model was generated in which the two LxxLL motifs of MED1 were both mutated to LxxAA to inhibit MED1's interaction with ER[80]. Interestingly, when the LxxLL motifs of MED1 were mutated, mice were born at appropriate Mendelian ratios, and both the heterozygous and homozygous mutants were generally healthy and fertile. The primary phenotype was in the mammary glands of homozygous MED1-LxxAA mutant knockin mice during the pubertal development. These mice showed a significant delay in mammary gland development as measured by the mammary ductal branch morphogenesis in both ductal length and number of branches[80]. On a cellular level, it was found that MED1 was primarily expressed in the luminal but not basal cells of the mammary gland. Significantly, it was found that the luminal progenitor cells were significantly decreased in MED1 mutant knockin mice despite MED1 being expressed at equal

levels. Importantly, while total estrogen levels and estrogen-dependent uterine growth were unaffected by the MED1 mutations, the mammary gland ductal growth in response to estrogen was greatly blocked as measured by growth and ER target gene expression.

When these MED1-LxxLL mutant knockin mice were further crossed with a HER2-driven mammary tumor model (MMTV-HER2), it was found that MED1 LxxLL mutations were able to significantly delay the tumor onset, growth and metastasis[81]. When compared to WT tumors, tumors from MED1 mutant knockin mice crossed with MMTV-HER2 mice showed impaired estrogen responsiveness and had much lower growth rates and metastasis. It was found that MED1 LxxLL motifs mutations significantly decreased cell proliferation, angiogenesis and cancer stem cell formation of MMTV-HER2 tumors. Consistent with these findings, the expression of a number of key ER/HER2 downstream genes involved in these processes (e.g., IGF-1, Cyclin-D1, LIF, ACP6, Twist, MMP9, VEGFA) were significantly inhibited by the MED1 mutations. Interestingly, we found that MED1 acts by directly regulating the ER downstream IGF-1 but not amphiregulin signaling pathway in our rescue experiments. This was further supported by several other findings including marked reductions in phosphorylation of IGF-1 signaling pathway proteins including the IGF-1 receptor, AKT, and mTOR in MED1 mutant tumors, phenocopying of IGF-1R inhibition and MED1 mutations in both mouse and human cancer cells, and correlation of MED1 and IGF-1 protein levels in human clinical samples. Overall, these studies support that MED1 and its LxxLL motifs play a central role in mammary tumorigenesis and suggest that MED1 could potentially serve as a tissue-specific target in breast cancer therapy.

ROLE OF MED1 IN ANTI-ESTROGEN TREATMENT RESISTANCE

As discussed above, MED1 overexpression is a frequent occurrence in approximately 50% of all breast cancers. Interestingly, the MED1 gene is located in the HER2 amplicon and coamplifies with HER2 in almost all instances. We have further confirmed that MED1 protein levels highly correlate with HER2 status of human breast cancer by using a human breast cancer tissue microarray[82]. Given HER2 amplification and overexpression is a common mechanism of endocrine resistance, Cui *et al.*[82] carried out to investigate whether HER2 and MED1 cooperate in driving endocrine resistance. Interestingly, it was found that MED1 levels and especially the phosphorylated form of MED1 are increased in tamoxifen-resistant BT474, MCF-7/TAM, MCF-7/HER2 cells compared to MCF-7 controls, in a manner dependent on HER2 and MAPK activation. Importantly, knockdown of MED1 in these cells was found to increase the sensitivity of these otherwise resistant cells to tamoxifen treatment. In addition to reduced expression of traditional ER target genes (e.g., TFF1, Cyclin D1) by MED1 knockdown in these cells, it was found that MED1 is also required for the expression of another class of newly defined HER2 activated ER-target genes like *LIF* and *ACP6*[82,83].

Mechanistically, it is known that when tamoxifen binds to ER, the conformational change forces ER into a repressive state, and rather than recruiting coactivators, ER preferentially recruits co-repressors such as NCoR and SMRT to inhibit transcription of downstream target genes[15]. However, in these tamoxifen-resistant cells with MED1 overexpression and activation, it was found that tamoxifen treatment still resulted in the recruitment of MED1 and phospho-MED1 but not co-repressors to the ER target gene promoters [Figure 2]. Importantly, this was completely reversed with MED1 knockdown in these cells and restored the recruitment of the co-repressors under tamoxifen treatment. Furthermore, it was found that only wild-type but not phospho-defective mutant of MED1 (T -> A) was able to displace the co-repressors after tamoxifen treatment in these MED1 knockdown cells, supporting the importance of MED1 phosphorylation by HER2 in mediating tamoxifen resistance.

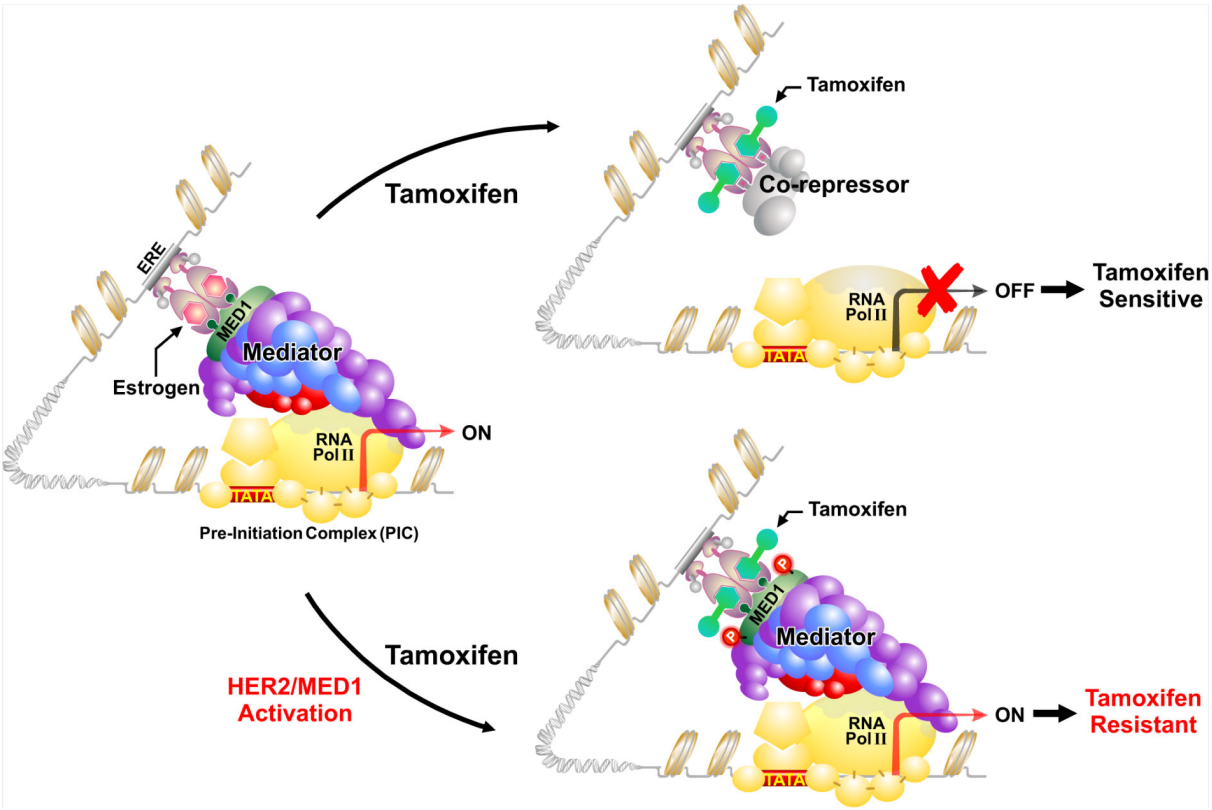

Figure 2. Molecular mechanisms of HER2 and MED1 in anti-estrogen treatment resistance. In endocrine-sensitive breast cancer cells, treatment with anti-estrogen tamoxifen results in the recruitment of co-repressors to suppress gene transcription. However, when MED1 is overexpressed and phosphorylated by growth factor cascades such as HER2, the MED1/Mediator complex rather than ER co-repressors is recruited to activate gene transcription and render endocrine resistance. Adapted from Leonard *et al.*[72], JZUS-B.

Consistent with our studies above, other laboratories have also reported clinical evidence indicating MED1 as a gene associated with poor endocrine treatment outcome, and that high MED1 expression correlates with poor survival of breast cancer patients that have undergone anti-estrogen therapy[84,85]. In addition, another genome-wide study identified an increased frequency of MED1 mutations in circulating plasma DNA in breast cancer patients following anti-estrogen and anti-HER2 treatments[86]. These studies suggest a broad role of MED1 in anti-estrogen treatment resistance to other anti-estrogen therapies besides tamoxifen. Indeed, we found that MED1 depletion sensitized the MCF-7 cell derived fulvestrant resistant cell line (MCF-7-F) and HER2/MED1 overexpressing BT-474 and ZR-75.1 cells to fulvestrant treatment as well *in vitro* and *in vivo*[87]. Together, these data support that MED1 overexpression and activation by growth factor signaling pathways like HER2 can drive broad resistance to anti-estrogen treatments, and that MED1 could serve as a potential therapeutic target to overcome such treatment resistance.

TARGETING MED1 IN ANTI-ESTROGEN RESISTANCE BREAST CANCER BY RNA NANOTECHNOLOGY

Based on these findings, Zhang's laboratory has developed a targeted approach to block MED1 specifically in anti-estrogen resistant breast cancers using an innovative RNA nanotechnology approach[88]. The reason we selected this approach is due to many advantages of the RNA nanotechnology: multifunctional capabilities with simultaneous targeting and therapy, high stability and nanoscale size allows slower body clearance and better tissue penetration, retention, and cellular uptake, low toxicity and immunogenicity,

controlled synthesis and self-assembly for great manufacturing and scale-up compatibility, *etc.*[88-93]. Furthermore, this technology also allows for the development of ways to target the transcriptional coactivators like MED1 that are difficult to target with traditional small molecules or antibody-based approaches. By using a phi29 3-way junction pRNA nanodelivery system, we have created RNA nanoparticles that are composed of two MED1-targeting siRNAs to silence MED1 and an aptamer that specifically binds to membrane receptor HER2 to allow for targeted delivery into breast cancer cells [Figure 3]. These pRNA-HER2apt-siMED1 RNA nanoparticles can be generated uniformly with high reproducibility as measured by dynamic light scattering and atomic force microscopy. By using 2'Fluoro modified nucleotides, these RNA nanoparticles exhibited high stability, even in conditions that mimic or are more extreme than physiological conditions, including treatment with RNase A, Serum, Urea or heat up to 70 degrees Celsius. Importantly, we have shown that treatment with recombinant Dicer enzyme can easily release the siRNA arms from the nanoparticle, an essential step in the RNAi pathway.

These highly stable multifunctional RNA nanoparticles have been successfully tested in both *in vitro* and *in vivo* preclinical models and exhibited highly desirable tumor-specific targeting and treatment efficacy with no apparent toxicity[88]. It was found that the pRNA-HER2apt-siMED1 nanoparticles can silence MED1 expression in both de novo tamoxifen-resistant BT-474 cells that have HER2 amplification and acquired tamoxifen-resistant MCF-7/TAM cells with elevated HER2 expression but no amplification. These knockdown effects are specific and dependent on both siMED1 sequences and intact HER2 binding aptamer. Furthermore, triple-negative breast cancer MDA-MB-231 cells were not able to take up these RNA nanoparticles or knockdown MED1. In addition to growth inhibition, we found that these MED1 targeting RNA nanoparticles significantly reduce cell migration, stem cell formation, and MED1 target gene expression compared to control scramble RNA nanoparticles or tamoxifen alone. When we cotreated these cells with tamoxifen, we found further decreased cell growth, motility, and cancer stem cell formation compared to either treatment alone.

The distribution and the efficacy of pRNA-HER2apt-siMED1 nanoparticles were further examined *in vivo* using a human breast cancer orthotopic xenograft mouse model. It was found that pRNA-HER2apt-siMED1 but not the HER2apt mutant nanoparticles were able to significantly accumulate and enrich in the tumors while both showed only residual levels in the liver and kidneys, and were undetectable in other organs such as lung, spleen and heart. Remarkably, we found that once a week systemic injection of the RNA nanoparticles through tail vein achieved greater tumor growth inhibition *in vivo* than regular 5 days per week tamoxifen treatment. Immunochemistry staining and western blot analyses confirmed that the expression of MED1 was significantly depleted in the pRNA-HER2apt-siMED1-treated groups along with a great reduction of Ki-67 expression, tumor growth, lung metastasis and stem cell formation compared to those of control treatments. Importantly, we did not observe apparent toxicity, and there was no body weight change or histological abnormalities in major organs during the course of treatment including liver and kidney. This lack of toxicity in these organs could be due to one or a combination of the rapid tissue clearance of the RNA nanoparticle, our tumor-specific HER2 aptamer ligand design and the inability of RNA nanoparticles to enter the cells in these organs, as well as the tissue-specific roles of MED1 and its lack of key biological functions in these organs, *etc.* Together, these findings support that our biosafe pRNA-HER2apt-siMED1 nanoparticle represents a highly promising new therapeutic regimen for potential future breast cancer treatment to overcome resistance.

MED1 IN HER2-MEDIATED TUMORIGENESIS AND ANTI-HER2 THERAPY RESPONSE

We have discussed above that MED1 is frequently overexpressed and coamplified in breast cancers with HER2 and crosstalk with HER2 in mediating anti-estrogen treatment resistance. However, it was still not

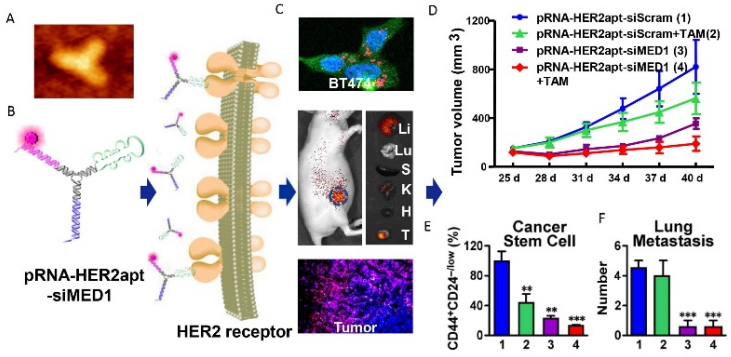

Figure 3. Overcoming breast cancer therapeutic resistance by MED1 targeting multifunctional RNA nanoparticles. The figure shows the atomic force microscopy (A) and schematic (B) of the pRNA-HER2apt-MED1siRNA nanoparticle, its tumor-specific uptake *in vitro and in vivo* (C), and therapeutic effect to inhibit endocrine-resistant tumor growth (D), cancer stem cell formation (E), and lung metastasis (F) *in vivo* in an orthotopic xenograft mouse model. $^{**}P < 0.01$, $^{***}P < 0.001$. Adapted from Zhang *et al.*[88], ACS Nano.

known whether MED1 plays a role in mediating HER2-mediated tumorigenesis and the response to anti-HER2 treatment. To achieve that, we have generated a MED1 mammary specific overexpression mouse model MMTV-MED1[55]. We found that over-expression of MED1 in mammary glands resulted in a slightly increased number of mammary stem and progenitor cells, but no other phenotypical abnormalities or increase in cancer incidence. However, when we crossed these MMTV-MED1 mice with the MMTV-HER2 mammary tumor-prone mouse model, we found a strong increase in mammary tumor formation marked both by the earlier onset of an initial tumor and an increase in the number of individual tumors formed per mouse. Furthermore, these MMTV-HER2/MMTV-MED1 tumors grew much faster and formed more lung metastases compared to MMTV-HER2 tumors. These *in vivo* findings were further validated by *in vitro* assays showing that isolated MMTV-HER2/MMTV-MED1 tumor cells had higher tumor mammosphere forming ability and metastatic capabilities. Flow cytometric analysis using stem cell markers and limiting dilution assays further indicated a higher stem-like cell content in MMTV-HER2/MMTV-MED1 tumors compared to that of MMTV-HER2 tumors.

Interestingly, MED1 was found to play a role in the tumor response to anti-HER2 treatment as well. It was found that MMTV-HER2/MMTV-MED1 tumors were highly resistant to anti-HER2 lapatinib treatment, as these tumors, despite treatment with lapatinib, still grew at the same rate as vehicle-treated HER2+ tumors and significantly faster than lapatinib treated HER2+ tumors[55]. Furthermore, MMTV-HER2/MMTV-MED1 tumors could still readily metastasize to the lung after lapatinib treatment, while the same treatment eliminated lung metastasis of MMTV-HER2 tumors. This is consistent with high Ki67 staining and the number of tumor-initiating cells by limiting dilution assays observed in MMTV-HER2/MMTV-MED1 tumors compared to that of MMTV-HER2 tumors after the treatment. Similar phenomena were also observed using human breast cancer cells and further supported the role of MED1 in HER2-treatment responses. Additional work is ongoing to test the role of MED1 in resistance to other anti-HER2 small molecule and antibody therapies discussed above. Mechanistically, it was found that MED1 overexpression results in increased expression of a variety of downstream genes, including JAB1, which regulates protein ubiquitination. Interestingly, MED1 was found to be a target of JAB1 directed ubiquitination as well, which was found to be essential for MED1's cyclic recruitment to its target genes' promoters and the expression of these target genes. Importantly, it was found that MED1 and JAB1 protein levels were closely correlated, particularly in HER2 positive breast cancer clinical samples[55]. Together, these data indicate that MED1 plays critical roles in HER2-mediated tumorigenesis and therapy response, and MED1 therapy could be

potentially utilized in the treatment of HER2+ breast cancers as well.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite the development and advancement in targeted therapies for ER+ and HER2+ breast cancers, severe unwanted side effects, frequent resistance, and relapse remain the greatest clinical challenges. In this review, we have provided experimental and clinical evidence from our laboratory and others that support the key roles of MED1 in both anti-estrogen and anti-HER2 treatment resistance and the potential use of MED1 therapy to overcome treatment resistance. It will be important in the future to further understand the molecular mechanisms underlying MED1 functions and its upstream and downstream regulators in mediating treatment resistance, as these will not only help us better understand the basic cancer biology of these tumor subtypes but also provide potential new therapeutic targets. In ER+ breast cancers, it will be important to understand what drives MED1 overexpression, the further fundamental molecular details on how MED1 regulates ER target gene expression, and whether targeting MED1 could delay the development of anti-estrogen treatment resistance, *etc.* It will also be interesting to know further the role and molecular mechanisms of MED1 in the treatment resistance of HER2+ tumors to both anti-HER2 small molecules and antibodies, whether it plays a role in mediating the poor response of ER+ tumors in HER2+ heterogeneous tumors, and what role it might play in HER2+ER- tumors and the mechanisms, among others.

Many of these questions should be addressed in appropriate breast cancer cell lines, organoids, orthotopic xenograft, and PDX models, and most importantly, tested in clinics. Significantly, we have developed an innovative patented RNA-nanotechnology-based approach to target MED1 specifically in breast cancer cells and successfully tested it in both *in vitro* and *in vivo* preclinical models. Given the many advantages of RNA nanotechnology described above and our highly stable multifunctional RNA nanoparticles designed with specific tumor targeting and dual MED1 siRNA therapeutic modules, we expect our MED1 RNA therapeutics to be less toxic, more effective, and less prone to develop treatment resistance. Since MED1 functions far downstream in the ER and HER2 pathways at the last step before transcription initiation, targeting MED1 will not only help gain better treatment outcomes but will likely be less prone to the development of resistances commonly occurring through aberrant activation of upstream signaling kinases or mutations in ER itself. Finally, the better safety profile and long-lasting effects with fewer treatments needed for our RNA nanoparticles could also mean better patient treatment compliance as well which is also a major issue with current treatment. With increasing FDA approval, large-scale production and broad use of RNA-based vaccines and medicines, we are very excited about the opportunities and fully anticipate that our RNA nanotherapeutics could represent a highly promising next-generation therapy to ultimately benefit patient care through better efficacy, fewer side effects, and improved patient quality of life and treatment compliance.

DECLARATIONS

Authors' contributions

Drafting, revising, and approving the article for submission: Bick G, Zhang J, Lower EE, Zhang X

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Multicellular biomarkers of drug resistance as promising targets for glioma precision medicine and predictors of patient survival

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Abstract

Aim: This study aimed to translate a known drug-resistance mechanism of long-term CSF1R inhibition into multicellular biomarkers that can serve as potential therapeutic targets as well as predictive markers for the survival of glioma patients.

Methods: Using existing data from a published mouse study of drug resistance in immunotherapy for glioma, we identified multicellular differentially expressed genes (DEGs) between drug-sensitive and drug-resistant mice and translated the DEGs in mouse genome to human homolog. We constructed correlation gene networks for drug resistance in mice and glioma patients and selected candidate genes via concordance analysis of human with mouse gene networks. Markers of drug resistance and an associated predictive signature for patient survival were developed using regularized Cox models with data of glioma patients from The Cancer Genome Atlas (TCGA) database. Predictive performance of the identified predictive signature was evaluated using an independent human dataset from the Chinese Glioma Genome Atlas (CGGA) database.

Results: Fourteen genes (*CCL22*, *ADCY2*, *PDK1*, *ZFP36*, *CP*, *CD2*, *PLAUR*, *ACAP1*, *COL5A1*, *FAM83D*, *PBK*, *FANCA*, *ANXA7*, and *TACC3*) were identified as genetic biomarkers that were all associated with pathways in glioma progression and drug resistance. Five of the 14 genes (*CCL22*, *ADCY2*, *PDK1*, *CD2*, and *COL5A1*) were used to construct a signature that is predictive of patient survival in the proneural subtype GBM patients with an AUC under the time-dependent receiver operating characteristic (ROC) of 2-year survival equal to 0.89. This signature also shows promising predic-

tive accuracy for the survival of LGG patients but not for non-proneural type GBMs.

Conclusion: Our translational approach can utilize gene correlation networks from multiple types of cells in the tumor microenvironment of animals. The identified biomarkers of drug resistance have good power to predict patient survival in some major subtypes of gliomas (the proneural subtype of GBM and LGG). The expression levels of the biomarkers of drug resistance may be modified for the development of personalized immunotherapies to prolong survival for a large portion of glioma patients.

Keywords: Drug resistance, tumor microenvironment, translational research strategy, multicellular gene correlation network, glioma, precision medicine

INTRODUCTION

Glioma is an aggressive and malignant brain tumor with a poor prognosis. The traditional standard-of-care therapies (surgical removal, radiotherapy, chemotherapy, *etc.*) only slightly extend the survival of glioma patients[1]. Despite the recent advances in cancer immunotherapies and targeted therapies in treating many types of cancer, only a fraction of patients developed durable responses, which indicates the common existence of intrinsic/acquired resistance to existing immunotherapies. To date, the effect of immunotherapies in treating glioma has been even more disappointing, partly due to prevalent drug resistance[2–4]. To improve patient survival, it is critical to discover potential therapeutic targets and prognostic biomarkers for novel biological interventions to overcome drug resistance.

The tumor microenvironment (TME) plays a crucial role in the progression and responses to therapies[5]. In addition to tumor cells (TCs), the TME also includes T cells, tumor-associated macrophages (TAMs), epithelial cells, *etc.*[6,7] The immunosuppressive action of TAMs based on the release of anti-inflammatory cytokines within the TME could promote the proliferation of tumor cells and the subsequent drug resistance[5,6,8,9]. Immunotherapies are often designed to enhance antitumor capacity of the immune cells such as TAMs, and, in turn, the enhanced TAMs could attack and kill TCs. In particular, inhibition of CSF1R (by the small-molecule BLZ945 treatment) in TAMs has been a promising intervention for glioblastoma (GBM) in mice; however, persistent usage of CSF1R inhibition can lead to drug resistance in mice[1]. Importantly, a well-designed mouse study published by Quail *et al.*[1] discovered and characterized the mechanism of the drug resistance to CSF1R inhibition in mice. Specifically, long-term inhibition of CSF1R in TAMs resulted in the increased secretion of IGF1 to TME and the alternative activation of TAM, which was reflected by the elevated expression level of M2-like genes. The combination of IGF1 in TME and its receptor in TCs, IGF1R, activated the downstream PI3K signaling pathways to support tumor regrowth and led to drug resistance. Based on this drug-resistant mechanism, they further identified multiple interventions, including blockage of IGF1R (by OSI906) and inhibition of PI3K pathway (by BKM120), that resulted in substantial improvement in survival in mouse studies when combined with CSF1R inhibition. However, the important findings from this mouse study of drug resistance have not been translated to human gliomas to prolong patient survival. Thus far, it is unclear whether the findings of the mice study can be successfully translated to some subtypes or all types of gliomas in humans.

Given the importance of the multiple types of cells in TME for drug resistance, it is desired to have cell-specific (immune cell and tumor cell) gene-expression data to investigate cell-specific effects and interactions between different types of cells in TME when studying drug resistance in humans. However, due to the extensive labor cost and technical challenges in obtaining cell-specific data in humans, discovering the multicellular mechanism of drug resistance directly in human trials is currently challenging. In contrast, as cell-type-specific gene expression data from mouse studies are more affordable[1], we suggest a translational study strategy that projects the multicellular results of the animal experiment to human genome to investigate drug resistance. In

particular, borrowing strength from the mouse study published by Quail *et al.*[1], we can combine cell-specific mouse gene expression data with gene expression data from human bulk tissue to identify biomarkers of drug resistance and patient survival. Furthermore, as Quail *et al.*[1] also identified interventions to overcome the drug resistance in mice, any genetic biomarkers we identify would likely to be actionable targets for therapeutic intervention in human precision medicine, too.

For the purpose of developing novel treatment targets that are feasible for biomedical intervention, for convenience, we would like to select a small set of genes that can adequately account for drug resistance as well as patients' survival. However, response and resistance to an intervention typically involve a great number of genes and pathways in addition to population heterogeneity. In practice, it is hard to decide which genes are biologically more important than others, given the vast number of genes involved, and it is generally challenging to distinguish driver genes from passenger genes based on cross-sectional gene expression data. To the best of our knowledge, there is no known method that can efficiently identify biomarkers of drug resistance with high predictive accuracy for patient survival. Given that biological pathways involve the cooperation of clusters of highly correlated genes, we used gene correlation network analysis and gene-set enrichment analysis to detect biologically important gene clusters. Focusing on gene clusters in important pathways can borrow strength from existing biological knowledge based on independent studies; thus, it should be more likely to determine genes with driver effects and avoid the abundance of false positives, compared to the common approach of focusing on the analysis of individual genes with top *P*-values. Moreover, important and well-connected genes in gene networks are generally sparse[10,11]; thus, constructing weighted gene networks reflecting such sparsity would be desirable. Moreover, regularized Cox regression models that account for the sparsity of important genes in correlation networks can be used to further shrink the number of candidate genes in order to form a compact gene set predictive of patients' survival.

In cancer research, the "one treatment for all patients" approach is generally impractical given various heterogeneities associated with cancers. For precision medicine, it is desired and more practical to find an effective and suitable treatment strategy for each particular subtype of cancer and subgroup of patients. Furthermore, it is important to identify treatment targets and biomarkers that have high prognostic accuracy for each specified subgroup of patients in order to develop novel personalized treatments including overcoming drug resistance in existing therapies. Indeed, complex diseases are often classified into subtypes characterized by the difference in histology and pathology. In particular, gliomas are usually classified into two major categories according to the World Health organization (WHO) grading: lower-grade gliomas (LGG; WHO grade II and III gliomas) and glioblastoma multiforme (GBM; WHO grade IV gliomas). GBM can be further divided into four subtypes based on their gene expression profile: classical, mesenchymal, proneural, and neural[12]. Due to the heterogeneity in histology and gene expression, drug resistance for different types of patients can be due to many different biological mechanisms involving many different pathways. Given the poor overall survival of GBM and gliomas currently, it is very valuable if we can identify a particular subgroup of patients that may benefit from interventions based on the identified drug-resistant targets or pathways.

In this study, we used a translational research strategy to identify biomarkers of drug resistance as targets for precision medicines for gliomas in humans. Beginning with results and data from an existing mouse study[1], we compared the gene expression levels between drug-sensitive and drug-resistant mice to obtain differentially expressed genes (*DEGs*) in TAMs and TCs, respectively, which were then translated to human homolog. Because the mouse study was conducted on mice initiated with the proneural subtype of GBM tumor, we hope the findings of the mouse study can be translated to the proneural type GBMs in humans. Thus, our subsequent analysis will first be conducted on the proneural type GBM subjects. Next, weighted gene correlation networks of drug resistance were constructed in TAMs and TCs, using the expression data of humans and mice, respectively. Then, we performed concordance analysis to compare human networks to mouse networks within each cell type and performed enrichment analysis to get the biologically important gene clusters

(indicating pathways), from which candidate genes were selected according to their importance and individual predictive capacity for patient survival. Lastly, integrating the findings of M2-like genes and PI3K pathways identified in the drug-resistance mouse study, we applied regularized Cox regression models to get a small set of genetic biomarkers. For precision medicine, it is important to identify some major subgroups or subtypes of gliomas such that expression levels of the identified molecular biomarkers of drug resistance can predict population survival rates of human glioma patients, and ideally, the expression levels of the biomarkers can be modified to prolong survival of a large portion of patients. Towards this end, time-dependent ROC curves, corresponding AUCs, and Kaplan-Meier (KM) curves were generated to demonstrate the predictive performance of the identified genetic biomarkers in the proneural subtype of GBM, non-proneural type of GBM, and LGG patients, respectively. The identified genetic biomarkers showed high AUCs at two years in the proneural subtype of GBM, indicating good predictive performance of the identified signature. Importantly, the signature developed using the proneural type mouse study had poor predictive power of survival in non-proneural subtypes of GBM, suggesting that different mechanisms and therapeutic targets should be considered for different subtypes of glioma. We also discuss the identified biomarkers as potential treatment targets to overcome drug resistance.

METHODS

A translational strategy to identify predictive biomarkers of drug resistance and patient survival

Since obtaining cell-specific gene expression data in human brains is a challenge and such cell-specific mouse data are available from the study by Quail *et al.*[1], we introduced a translational study design that borrows strength from the mouse cell-specific data to identify biomarkers of drug resistance in humans. First, we identified and translated DEGs between drug-resistant (Reb) and drug-sensitive (Ep) mice to humans in TAMs and TCs, respectively. Then, weighted gene correlation networks were constructed, and gene clusters were detected for TAMs and TCs in mice and human patients, respectively. By comparing mouse networks to human networks via concordance analysis, biologically important gene clusters were selected from the highly concordant gene clusters, integrating the result from enrichment analysis. Next, to discover therapeutic targets of gliomas that may be actionable in future intervention, we reduced the number of candidate genes using principal component analysis (PCA) and K-index based on the biologically important gene clusters. In addition, since M2-like genes and PI3K pathway-related DEGs were indicated to be associated with drug resistance in mice[1], they were combined with genes selected from the biologically important gene clusters to construct predictive signatures using regularized Cox regression models. Finally, the performance of the identified predictive signature was examined by KM analysis and time-dependent ROC curves. The entire workflow is shown in Figure 1. More details are described in the following subsections.

An existing randomized mouse study for drug resistance in gliomas

To investigate the biological mechanism of drug resistance to the CSF1R inhibition of TAMs, a randomized study of mice with gliomas was conducted and reported by Quail *et al.*[1]. The CSF1R inhibition treatment is aimed at enhancing immune capacity of the tumor-associated macrophage (TAM) so that the treated TAMs can more effectively kill glioma cells or inhibit tumor growth. There were two randomized groups of mice in the study conducted by Quail *et al.*[1]: the treatment naïve or vehicle group (Veh) and the treatment group or CSF1R inhibition group. The treatment group was divided into two subgroups: the group of treated mice that had durable treatment response, called the drug-sensitive or endpoint (Ep) group, and the group of treated mice that had tumor regrowth after short-term treatment response, called the drug-resistant or rebound (Reb) group. There were five Veh samples, six Ep samples, and four Reb samples with available gene expression data (RNA-seq data) for both TCs and macrophages (TAMs). The RNA-seq data of the 15 samples were selected for subsequent analyses, and the data could be downloaded from the Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE69104. By comparing gene expressions in TAMs of the treated and untreated (Veh) groups, we could identify differentially expressed genes (DEGs)

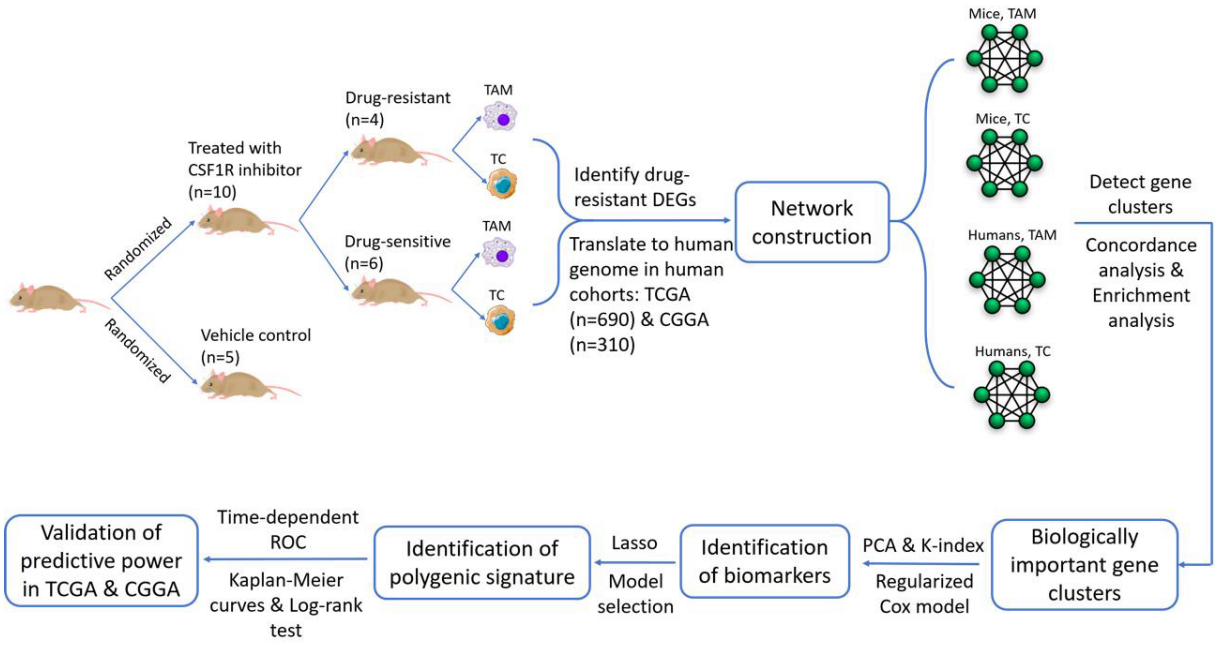

Figure 1. Flowchart of the translational study design. TAM: Tumor-associated macrophages. TC: Tumor cells. DEGs: Differentially expressed genes. TCGA: The Cancer Genome Atlas. CGGA: Chinese Glioma Genome Atlas. PCA: Principal component analysis. ROC: receiver operating characteristic.

that are modifiable by the CSF1R inhibition treatment. We could also construct gene networks in TAMs that are modified by the treatment. Furthermore, we could construct correlation gene networks for the treated mice by contrasting the drug-resistant (Reb) and drug-sensitive (Ep) groups in TAMs and in TCs, as discussed in subsequent sections.

Human glioma cohorts

To translate the identified differentially expressed genes (DEGs) and candidate markers of the drug resistance in mice to human glioma patients, two independent human glioma cohorts, The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) and Chinese Glioma Genome Atlas (CGGA) database (http://www.cgga.org.cn/), were prepared for the subsequent gene network construction, gene cluster detection, and survival modeling. After matching the clinical information with the gene expression data (RNA-seq data) for each patient, a set of 690 samples (GBM: n = 165; LGG: n = 525) from TCGA and a set of 310 samples from CGGA (GBM: n = 138; LGG: n = 172) were collected. Since the mice were initiated with tumors from the proneural subtype of GBM, the subsequent analyses including network construction and signature identification were mainly performed in GBM proneural patients (n = 38 in TCGA; n = 30 in CGGA). In general, the TCGA dataset was used as the training dataset for the identification of the prognostic signature, and the CGGA dataset was used as an independent testing set to validate the predictive power of the polygenic signature.

Differential gene expression analysis in mice and translation to human homolog

Differential gene expression analyses were conducted to compare the average gene expression level between drug-sensitive (Ep) and drug-resistant mice (Reb) for TCs and TAMs, respectively. The RNA-seq read counts data were normalized by the trimmed mean of M-values method. For each gene, the expression level was modeled by the generalized linear model with a negative binomial link, and the quasi-likelihood (QL) F-test was used to compare the gene expression level between the Ep and Reb subgroups. The logarithm of fold-change (logFC; to base 2) and nominal P-value were calculated using the R/Bioconductor software package

edgeR[13–15]. The Benjamini-Hochberg false discovery rate (FDR) was used as an adjustment for multiple testing. Genes with $|\log \mathrm{FC}| > 1.5$ and FDR < 0.05 were considered as differentially expressed genes (DEGs) for TCs and TAMs, respectively. Due to the fact that human gene expressions are derived from the RNA-seq data of bulk tumor tissues, we only focused on DEGs that had the same signs of logFC in both TCs and TAMs in mice. This facilitated the interpretations of concordance of up- or downregulations of candidate DEGs in humans and mice. We then translated the selected DEGs identified in mice to human homolog using the NCBI database (https://www.ncbi.nlm.nih.gov/gene), which resulted in 818 DEGs in TAMs and 1730 DEGs in TCs.

A network-based and translational research strategy to select candidate genes from important gene clusters

Our goal is to identify a set of key genes that has the potential as novel treatment targets to overcome drug resistance as well as is predictive of patient survival. The differential expression analyses typically discover a large number of DEGs, which makes it difficult in practice to design effective interventions for all of them in lab-based biological studies. Hence, we needed to refine the set of candidate DEGs to get a relatively smaller set of candidate genes that are indicative of drug resistance and predictive of survival. Depending on the co-expression network, DEGs can be clustered according to their intrinsic correlations. Clusters of genes may pertain to specific biological functions and have a greater impact on the outcome than single genes. In general, given a set of gene expression data, it is straightforward to construct correlation gene networks or weighted correlation gene networks, e.g., as done by Sun *et al.*[16] or He *et al.*[17]. In the following sections, we discuss how to identify key genes from important clusters detected through weighted gene correlation networks (WGCNA)[18].

Detection of gene clusters (modules) by weighted correlation network analyses for TC and TAM in mice and human

First, we constructed the weighted correlation network using weighted correlation network analyses (WGCNA) for TAMs and TCs, in mice and humans, respectively, which resulted in four networks. For each of the networks, denote the gene expression matrix as $X = [x_{ij}]_{n\times p}$, where n is the sample size, $i = 1, \cdots, n$, p is the number of genes, and $j = 1, \cdots, p$. Let $\boldsymbol{x}^{(j)} = (x_{1j}, \cdots, x_{nj})^{\mathrm{T}}$ denote the expression of the j^{th} gene and $\boldsymbol{x}_i = (x_{i1}, \cdots, x_{ip})$ denote the gene expression of the i^{th} subject. A correlation network is fully specified by its adjacency matrix $A = [a_{ij}]_{p\times p}$, which is a symmetric $p \times p$ matrix with entries in [0, 1] representing the connection strength of the i^{th} and j^{th} gene. The weighted adjacency a_{ij} is modeled by the power adjacency function, that is,

$$a_{ij} = s_{ij}^{\beta}, \tag{1}$$

where s_{ij} is the co-expression similarity that defined by the Pearson correlation, i.e.,

$$s_{ij} = |\operatorname{cor}(\boldsymbol{x}^{(i)}, \boldsymbol{x}^{(j)})| = \left| \frac{\sum_{k=1}^{\mathrm{n}} \left(x_{ik} - \bar{x}^{(i)}\right)\left(x_{jk} - \bar{x}^{(j)}\right)}{\sqrt{\sum_{k=1}^{\mathrm{n}} \left(x_{ik} - \bar{x}^{(i)}\right)^2 \sum_{k=1}^{\mathrm{n}} \left(x_{jk} - \bar{x}^{(j)}\right)^2}} \right|, \tag{2}$$

where $\bar{x}^{(i)}, \bar{x}^{(j)}$ are the mean expression level of the i^{th} and j^{th} genes. The power parameter $\beta (\beta \geq 1)$ was chosen by applying the approximate scale-free topology criterion. Details can be found in the work of Zhang and Horvath (2005)[19].

The network was constructed once β was specified. Next, we detected clusters of genes that were tightly interconnected. Such clusters of genes in the WGCNA method are called modules. To group the highly correlated genes into modules, we needed to introduce a distance measure that quantified the dissimilarity between each pair of genes within a weighted correlation network. We adopted the topological overlap matrix (TOM)-based dissimilarity, which is commonly used in many applications. The TOM-based dissimilarity is defined as

$$d_{ij} = 1 - \frac{l_{ij} + a_{ij}}{\min\{k_i, k_j\} + 1 - a_{ij}}, \tag{3}$$

where a_{ij} is the weighted adjacency defined in the weighted correlation network, $l_{ij} = \sum_u a_{iu}a_{uj}$, $k_i = \sum_u a_{iu}$. The hierarchical clustering dendrogram (tree) can be built with $\{d_{ij}\}_{i\neq j}^{p}$. Dynamic branch cut method was applied to identify gene modules from the hierarchical clustering dendrogram[20]. Parameters involved in the network construction and module detection were selected for each of the four networks individually (TAM network in humans, TC network in humans, TAM network in mice, and TC network in mice). The network construction and module detections were performed using the R/Bioconductor package WGCNA[18].

Identification of important gene clusters through concordance analysis between mouse and human modules

We identified four sets of modules from the weighted gene correlation network: modules for TAM in mice, modules for TC in mice, modules for TAM in humans, and modules for TC in humans. These modules can be viewed as "sub-networks" as they represent gene clusters in which genes are closely correlated. They may perform certain biological functions, since biological functions are rarely determined by a single gene, but rather by a set of tightly interconnected genes. In addition, the correlation networks and subsequently identified modules are based on DEGs that are differentially expressed between Reb mice and Ep mice. Thus, modules and their underlying biological functions identified in mice TAM and TC are likely to be associated with drug resistance. In each cell type, given that mouse modules and human modules share the same set of DEGs, and mice and humans are evolutionarily conserved, it would be of interest to know whether mouse modules and human modules perform similar biological functions or if the sub-networks and biological functions in mice are preserved in humans. If a mouse module and a human module do share a "sub-network", its underlying drug resistance-related biological functions should be more likely to be translated to humans. Therefore, in the same cell type, the concordance between each pair of mouse-human modules was assessed by calculating the number of genes that overlapped for each pair of mouse-human modules. Whether such overlap was due to chance alone was assessed by the Fisher's exact test. Contingency tables are reported for TC and TAM, respectively. Specifically, gene clusters from the top significantly associated mouse-human modules were selected by setting a threshold for *P*-values. In addition to the translation of drug-resistant DEGs from mice to humans at "gene-level", the concordance analysis between mouse modules and human modules can be viewed as a "network-level" translation, which is more relevant to reflect biological functions, since biological functions are normally activated by a set of genes instead of a single gene. Thus, adding "network-level" translation could help avoid false positives while enhancing the likelihood of success of the translational approach.

Enrichment analysis of important gene clusters

The biological functions of the gene clusters identified by the overlaps were investigated by the gene set enrichment analyses (GSEA) using Metascape[21] (http://metascape.org), which is a widely used online tool for gene annotation and enrichment analysis integrating multiple well-known ontology sources, including the KEGG Pathway, GO Biological Processes, *etc*. Gene clusters that are enriched in biologically relevant pathways were selected for the subsequent analyses. Gene set enrichment analysis leverages existing biological knowledge drawn from independent, published studies and databases, which helps to find biologically important gene clusters that are more relevant to the clinical outcome and reduce the likelihood of false-positive findings. Key genes can be further selected from the biologically important gene clusters. In short, using GSEA produces results that are more likely to be biologically meaningful and reproducible because it integrates biological and statistical information from other existing databases.

Selection of a small set of candidate genes from biologically important gene clusters

While several gene clusters that are biologically important for disease progression were selected by the enrichment analysis, these gene clusters still contained too many genes chosen as biomarkers of drug resistance and targets for possible interventions. Thus, we sought an even smaller set of candidate genes that are not only functionally important and representative for each of the selected gene clusters, but also possess good predictive accuracy for the survival of human glioma patients. Accordingly, two criteria were adopted to select such candidate genes. The first criterion was about the importance of the gene within each selected cluster. The first

principal component (PC) is a good summary metric for a given cluster, which is denoted as "eigengene"[18]. Assuming the eigengene is a good representative for a given cluster, for each gene, its correlation with the eigengene can be used to quantify its importance within a cluster. Higher correlations indicate stronger biological importance. Thus, the eigengene of the q^{th} selected cluster, denoted as $E(q)$, was calculated by the PCA. Similar to the concept of module membership, we defined the cluster membership as the Pearson correlation between the i^{th} gene and $E(q)$, that is,

$$CM_i^{(q)} = |\operatorname{cor}(\boldsymbol{x}^{(i)}, E(q))|. \tag{4}$$

Important candidate genes would be highly correlated to E(q) and can be selected by choosing an appropriate cut-off for $CM_i^{(q)}$.

The second criterion was about the predictive accuracy of survival. K-index is a commonly used metric that measures the overall concordance of a risk score and the survival, i.e., $P(T_1 > T_2 | R_2 > R_1)$, where T_j is the survival time and R_j is the risk score[22]. It does not depend on the censoring distribution, which makes it more general to assess the predictive power[23]. Higher K-index implies better predictive accuracy. Then, the K-index of the i^{th} gene was calculated by

$$K_i = \frac{2}{n(n-1)} \sum_{s \neq t} \frac{I(R_{i,t} > R_{i,s})}{1 + \exp(R_{i,t} - R_{i,s})}, \tag{5}$$

where $R_{i,j}$ is the linear combination of the covariates obtained from the univariate Cox regression model for the i^{th} gene and j^{th} subject, and $I(\cdot)$ is the indicator function. Genes that are predictive of survival would be selected by choosing an appropriate cut-off for K_i. In our study, candidate genes were selected by setting $CM_i^{(q)} > 0.7$ and $K_i > 0.55$ for each of the selected biologically important clusters.

Identification of the genetic biomarkers via regularized Cox regression model

As discussed in the previous section, we selected candidate genes from biologically important clusters according to cluster membership and K-index. However, the number of candidate genes heavily depends on the threshold chosen. A large number of genes can be selected when a low threshold is chosen. In addition, cluster membership and K-index are univariate methods, which do not take into account the correlation between genes within each cluster. Thus, the sparse-group lasso Cox regression model[24] was adopted to further shrink the number of candidate genes as biomarkers of drug resistance. It is a multivariate model that can account for sparsity and the correlation within clusters. Suppose p candidate genes belonging to m clusters were selected in previous steps, and their expressions for the i^{th} sample are denoted as $\boldsymbol{x}_i = (x_{i1}, \cdots, x_{ip})$. Let $\boldsymbol{x}_{i(l)}$ denote the gene expression of p_l genes in the l^{th} group and $\boldsymbol{\beta}_{(l)}$ denote the regression coefficient of $\boldsymbol{x}_{i(l)}$. Then, the coefficient $\boldsymbol{\beta}$ for $\boldsymbol{x}_i$ is estimated by

$$\hat{\boldsymbol{\beta}} = \arg\max_{\beta} \left\{ l_n(\boldsymbol{\beta}) - (1-\alpha)\lambda \sum_{l=1}^{\mathrm{m}} \sqrt{p_l} \|\boldsymbol{\beta}_{(l)}\|_2 - \alpha\lambda \|\boldsymbol{\beta}\|_1 \right\}, \tag{6}$$

where $\alpha \in [0, 1]$ is the weighting parameter for the combination of lasso and group-lasso penalties, λ is the tuning parameter, $l_n(\boldsymbol{\beta}) = \frac{1}{n} L_n(\boldsymbol{\beta})$, and $L_n(\boldsymbol{\beta})$ is the log-partial likelihood function:

$$L_n(\boldsymbol{\beta}) = \sum_{i=1}^{\mathrm{n}} \delta_i \left\{ \boldsymbol{x}_i^{\mathrm{T}} \boldsymbol{\beta} - \log \left[\sum_{j \in R(t_i)} \exp(\boldsymbol{x}_j^{\mathrm{T}} \boldsymbol{\beta}) \right] \right\}, \tag{7}$$

where (t_i, δ_i) is the observed survival time and censor indicator ($\delta_i = 1$ if the survival time is observed, $\delta_i = 0$ if the survival time is censored). The sparse group-lasso Cox regression was performed by the R package SGL[24].

According to Quail *et al.*[1], resistance to CSF1R inhibition was reflected by the elevated expression level of M2-like genes in TAMs and the activation of PI3K pathways in TCs. DEGs involved in M2-type activation and PI3K pathways are very likely to be associated with glioma survival. Therefore, we performed a sparse group-lasso (SGL) analysis to select genetic biomarkers from the combination of biologically important clusters and two additional groups of candidate genes: M2-like and PI3K pathway genes. α was set as 0.98 for more sparsity within the cluster. The tuning parameter λ was determined by 10-fold cross-validation.

Moreover, to obtain a parsimonious model, we further reduced the number of biomarkers using the L_1-Cox regression. The coefficients were estimated by

$$\hat{\boldsymbol{\beta}} = \arg\max_{\beta}\{l_n(\boldsymbol{\beta}) - \lambda\|\boldsymbol{\beta}\|_1\}, \tag{8}$$

where the tuning parameter λ is determined by cross-validation. The candidate genes with non-zero coefficients were selected as the prognostic signature for glioma.

Evaluation for the predictive performance of the constructed drug-resistant signature

The predictive accuracy of the signature identified in the previous section was evaluated in different subgroups using time-dependent receiver operating characteristic (ROC) analyses. In each subgroup, a Cox regression model was fitted using all genes in the final signature to obtain the regression coefficient $\tilde{\boldsymbol{\beta}}$ in the training set (TCGA). Risk scores were calculated by $\boldsymbol{x}_i^{\mathrm{T}}\tilde{\boldsymbol{\beta}}$ in both the training set and the testing set (CGGA). Given a cut-off $c \in R$, the sensitivity and specificity at a specific time t is

$$\begin{aligned} Se(c,t) &= P(\boldsymbol{x}_i^{\mathrm{T}}\tilde{\boldsymbol{\beta}} > c|\delta_i(t) = 1), \\ Sp(c,t) &= P(\boldsymbol{x}_i^{\mathrm{T}}\tilde{\boldsymbol{\beta}} \le c|\delta_i(t) = 0), \end{aligned} \tag{9}$$

where $\delta_i(t)$ is the censor indicator at time t. The time-dependent ROC curve[25] could be plotted by connecting all the coordinates $(1 - Sp(c,t), Se(c,t))$ at time t, and the time-dependent AUC at time t is

$$AUC(t) = \int_{-\infty}^{+\infty} Se(c,t)d[1 - Sp(c,t)]. \tag{10}$$

In our study, one-, two-, and three-year time-dependent AUCs in training set and testing set were calculated by R package timeROC[26].

In addition, patients could be further divided into a high risk of death group and a low risk of death group by taking the median of risk scores as a cut-off. KM curves were generated for the high-risk and low-risk groups of patients, and the log-rank test was employed to examine the significance of the difference in the overall survival between the high/low-risk groups.

RESULTS

Module detection from correlation networks constructed in mouse and human

Based on the translation of DEGs of drug resistance from mouse to human homolog, 818 DEGs were used to construct a correlation network for macrophages (TAMs), and 1730 DEGs were used for tumor cells (TCs), in both mice and humans. On the one hand, for the mouse TAM network, the soft threshold power parameter β was chosen to be 9 by applying the approximate scale-free topology criterion. Using the dynamic branch cutting method, setting the "deepSplit" parameter to be 3, "minClusterSize" parameter to be 30, and merging the highly correlated modules together, four modules were identified, as demonstrated in Figure 2A. Each branch referred to a gene and was marked by different colors, which represented different modules. Genes not assigned to any module were marked in grey. For the mouse TC network, the soft threshold power parameter β was chosen to be 16. Using the dynamic branch cutting method, setting the "deepSplit" parameter to be 3,

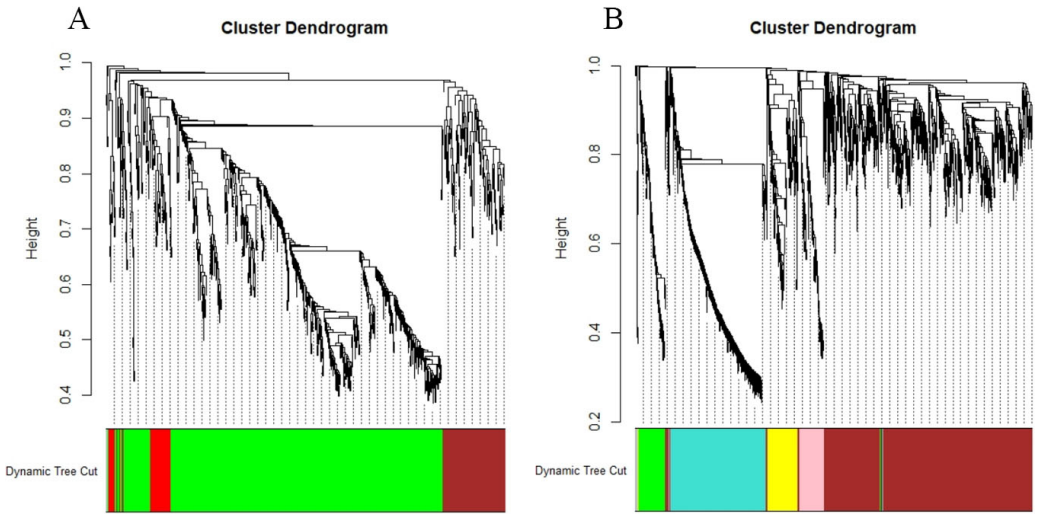

Figure 2. Hierarchical clustering dendrograms and modules identified in mice. (A) Dendrogram for TAM in mice. Four modules were identified (including the grey cluster, which represents genes that were not assigned to any cluster). (B) Dendrogram for TC in mice. Six modules were identified (including the grey module). Each branch refers to a gene and is marked by different colors, which represent different modules. The "height" axis refers to the value of the TOM-based dissimilarity.

"minClusterSize" parameter to be 50, and merging the highly correlated modules together, six modules were identified, including the grey module, demonstrated in Figure 2B.

On the other hand, the human TAM network was constructed using the WGCNA method on patients classified as the proneural subtype GBM using 818 DEGs translated from the candidate genes in TAMs of mice differentially expressed between drug-resistance and drug-sensitive subgroups of mice. The soft threshold power parameter β was chosen to be 6 by applying the approximate scale-free topology criterion. Using the dynamic branch cutting method (by means of dissimilarity matrix of mice), setting the "deepSplit" parameter to be 3, "minClusterSize" parameter to be 30, and merging the highly correlated modules together, seven modules were identified (including the grey module), as demonstrated in Figure 3A. The human TC network was constructed on patients classified as GBM proneural using 1730 DEGs. The soft threshold power parameter β was chosen to be 6. Using the dynamic branch cutting method, setting the "deepSplit" parameter to be 3, and "minClusterSize" parameter to be 70, eight modules were identified (including the grey module), as demonstrated in Figure 3B.

Selection of biologically important gene clusters from top-related human-mouse modules

Four modules were identified in the mouse TAM network, six modules in the mouse TC network, seven modules in the human TAM network, and eight modules in the human TC network. Since the mouse and human networks were constructed based on the same set of genes, we examined the similarities between them within the same type of cell. Thus, the contingency tables were generated to show the overlaps of each pair of mouse-human modules for TAM and TC, respectively, as shown in Figure 4A and B. The human modules with their sizes were spread as columns, and the mouse modules with their sizes included were spread as rows. In each cell, the number of overlapped genes for a given pair of mouse-human modules was calculated. Fisher's exact test was applied to test whether the overlap was statistically significant versus due to chance alone, and the P-value is shown in the parentheses (in $-\log_{10}$ scale). The color scale represents the P-value of the Fisher's exact test: the darker the color, the lower the p-value and the stronger significance of the overlap.

By setting a threshold for p-value $< 10^{-4}$, in Figure 4A, the top three most significant gene clusters overlapped between mice and humans for TAM are mouse brown-human green (MH-TAM1), mouse green-human turquoise

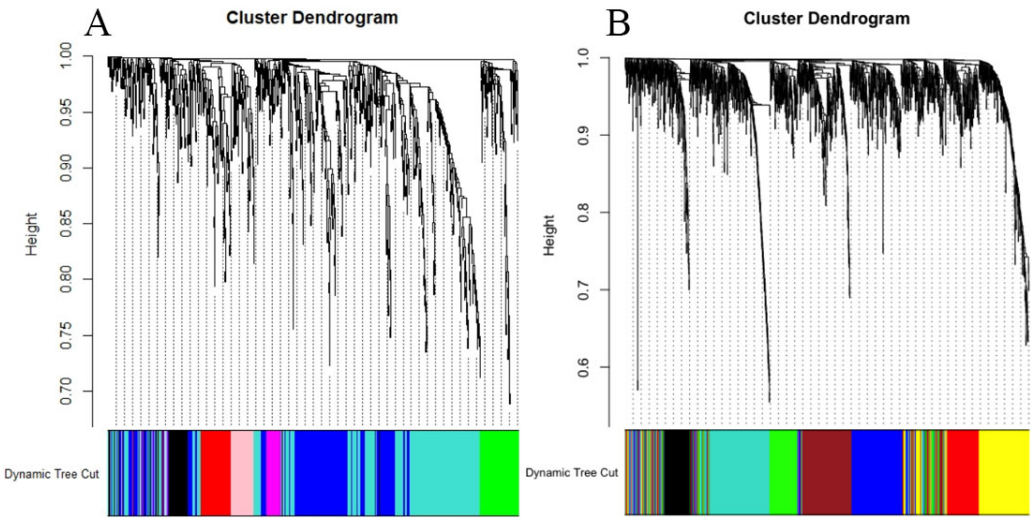

Figure 3. Hierarchical clustering dendrograms and modules identified in humans. (A) Dendrogram for TAM in humans. Seven modules were identified (including the grey cluster, which represents genes that were not assigned to any cluster). (B) Dendrogram for TC in humans. Eight modules were identified (including the grey module). Each branch refers to a gene and is marked by different colors, which represent different modules. The "height" axis shows the value of the TOM-based dissimilarity.

(MH-TAM2), and mouse brown–human black (MH-TAM3). In Figure 4B, the top four most significant gene clusters overlapped between mice and humans for TC are mouse green-human yellow (MH-TC1), mouse pink-human black (MH-TC2), mouse brown-human blue (MH-TC3), and mouse brown-human turquoise (MH-TC4). Genes in each of the seven clusters are highly correlated in both mice and humans, which makes the drug resistance more likely to be translated to humans from mice.

To see what biological impacts these overlaps might have, functional gene set enrichment analyses (GSEA) were performed on each of the seven clusters with significant overlaps. The results are shown in Figure 5A-G. Figure 5A-C shows that, in TAM, MH-TAM1 was enriched in GABA receptor signaling and cell cycle; MH-TAM2 was enriched in inflammatory response, lymphocyte activation, *etc*.; and MH-TAM3 was enriched in cellular responses to external stimuli. Figure 5D-G suggests that, in TC, MH-TC1 was enriched in microglia pathogen phagocytosis pathways, *etc*.; MH-TC2 was enriched in extracellular matrix organization *etc*.; MH-TC3 was enriched in mitotic cell cycle process, chromosome segregation, *etc*.; and MH-TC4 was enriched in synapse organization, neural system, *etc*. The inflammatory response, microglia pathogen phagocytosis pathways, extracellular matrix organization (ECM), mitotic cell cycle process, *etc*., are the most significantly enriched pathways and are believed to play an important role in cancer metabolism and progression. Specifically, inflammation increases susceptibility to cancer development and facilitates all stages of tumorigenesis [27]. Microglia is crucial in phagocytosing tumor cells [28]. In tumor tissues, the growth and malignancy of tumors as well as the response to therapy are affected by the ECM [29]. Thus, we mainly focused on the MH-TAM2, MH-TC1, MH-TC2, and MH-TC3 clusters for the subsequent identification of candidate genes and drug-resistant signatures.

Identification of the drug-resistant biomarkers and predictive signature of survival in the proneural subtype of GBM

We wanted to effectively link expression levels of candidate biomarkers of drug resistance to the population survival rate of human glioma patients. To obtain a relatively smaller but most important candidate set of genes for the identification of drug-resistant biomarkers, cluster membership (CM) and K-index were first calculated for each gene within each of the four biologically important gene clusters among proneural GBM patients. By setting the thresholds $CM > 0.7$ and $K > 0.55$, 105 genes were selected as functionally important

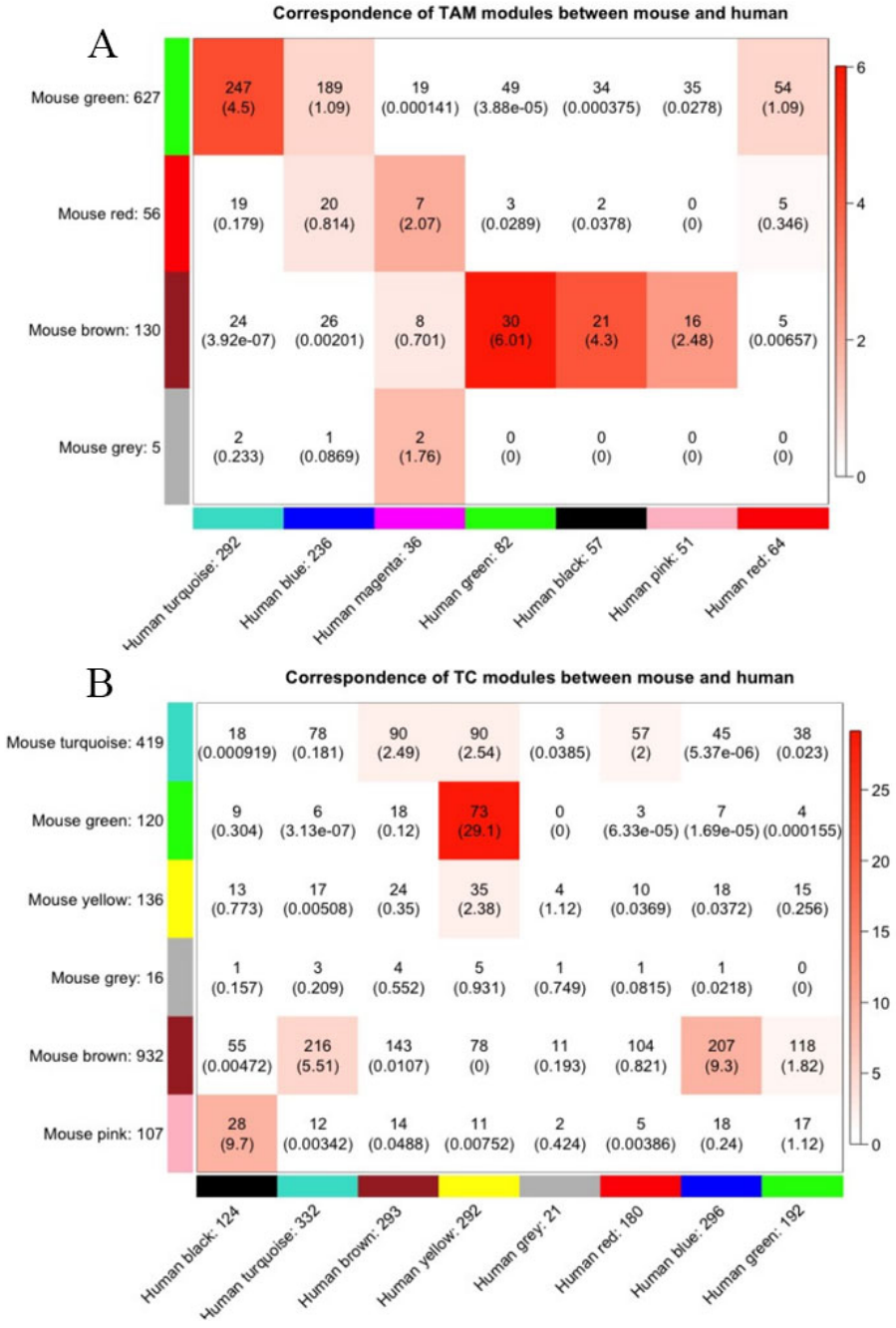

Figure 4. Correspondence between mouse and human modules: (A) correspondence of TAM modules between mouse and human; and (B) correspondence of TC modules between mouse and human. The human modules with their sizes were spread as columns, and the mouse modules with their sizes included were spread as rows. In each cell, the number of overlapped genes for a given pair of mouse-human modules was calculated, and the statistical significance of the overlap was tested by the Fisher's exact test. The *P*-value is shown in the parathesis (in $-\log_{10}$ scale). The color scale also represents the *P*-value of the Fisher's exact test: the darker the color, the lower the *P*-value and the stronger significance of the overlap. TAM: Tumor-associated macrophages. TC: Tumor cells.

and predictive of survival from the four selected biologically important clusters. In addition to the 105 genes, since M2-like genes and IGF/PI3K pathways were considered important for drug resistance in immunotherapy in animal models[1], 15 M2-like and 5 PI3K pathway genes that were also differentially expressed between Ep and Reb mice were added as two additional groups of genes. As a result, 125 candidate genes were prepared for the construction of prognostic signatures.

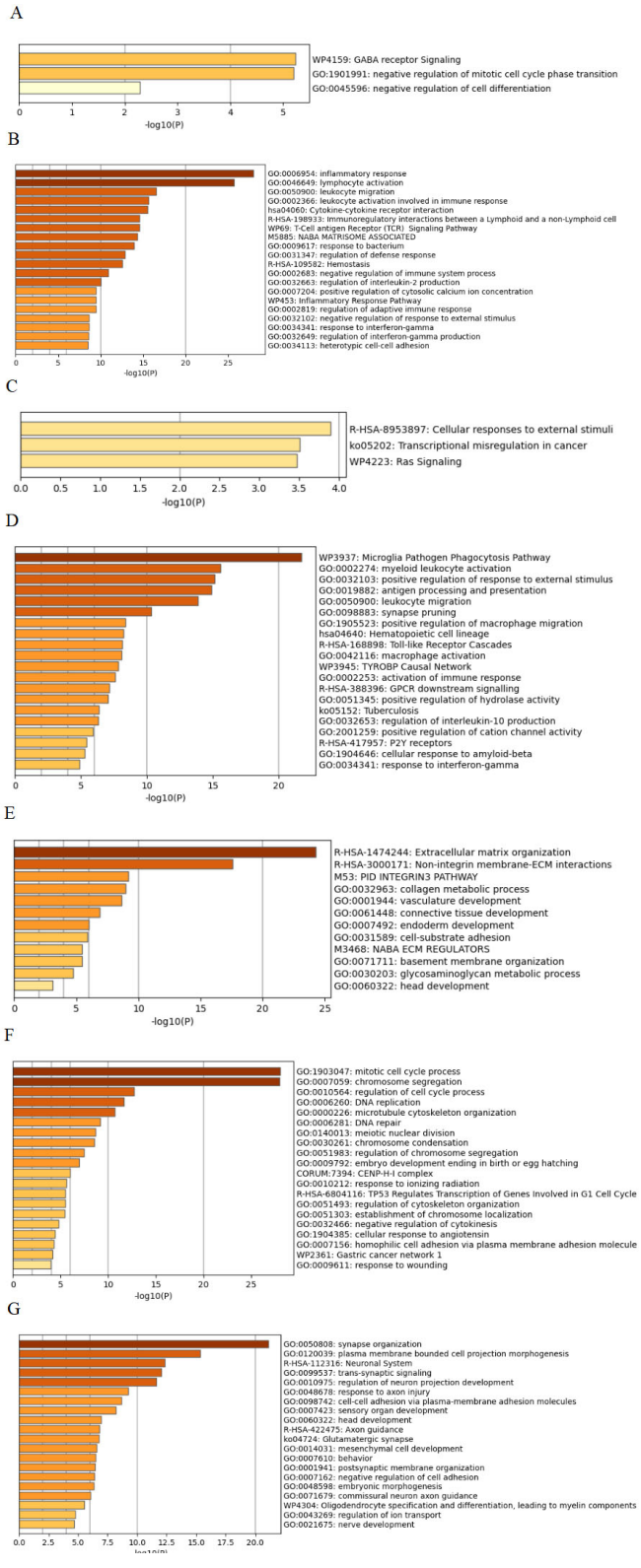

Figure 5. Functional enrichment analyses for the seven gene clusters overlapped between mouse and human: (A) mouse brown-human green in TAM (MH-TAM1); (B) mouse green-human turquoise in TAM (MH-TAM2); (C) mouse brown-human black in TAM (MH-TAM3); (D) mouse green-human yellow in TC (MH-TC1); (E) mouse pink-human black in TC (MH-TC2); (F) mouse brown-human blue in TC (MH-TC3); and (G) mouse brown-human turquoise in TC (MH-TC4).

Table 1. Summary for the 14 identified genetic biomarkers

Gene symbol	Gene name	Cell type	Gene clusters	Pathways
CCL22	C-C motif chemokine ligand 22	TAM	M2-like gene	Inflammatory response; response to cytokine
ADCY2	Adenylate cyclase 2	TC	PI3K pathway-related genes	PI3K pathways
PDK1	Pyruvate dehydrogenase kinase 1	TC	PI3K pathway-related genes	PI3K pathways
CP	Ceruloplasmin	TAM	MH-TAM2	Positive regulation of cytosolic calcium ion concentration
ZFP36	ZFP36 ring finger protein	TAM	MH-TAM2	Response to cytokine; leukocyte activation
CD2	CD2 molecule	TAM	MH-TAM2	Lymphocyte activation; positive regulation of cytokine production
PLAUR	Plasminogen activator, urokinase receptor	TAM	MH-TAM2	Regulation of leukocyte activation
ACAP1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	TAM	MH-TAM2	-
COL5A1	Collagen type V alpha 1 chain	TC	MH-TC2	ECM organization; PI3K pathways
FAM83D	Family with sequence similarity 83 member D	TC	MH-TC3	mitotic cell cycle, *etc.*
PBK	PDZ binding kinase	TC	MH-TC3	mitotic cell cycle, *etc.*
FANCA	FA complementation group A	TC	MH-TC3	mitotic cell cycle, *etc.*
ANXA7	Annexin A7	TC	MH-TC3	-
TACC3	Transforming acidic coiled-coil containing protein 3	TC	MH-TC3	mitotic cell cycle, *etc.*

Cell type (TAM or TC) refers to the cell from which the gene was selected. Gene clusters list the gene clusters to which the gene belonged. Pathways indicate the pathways that the gene was associated with (if any). TC: Tumor cells. TAM: Tumor-associated macrophages.

Next, considering both the correlation and the sparsity within each cluster, a sparse group-lasso was performed on the 125 candidate genes from six groups: MH-TAM2, MH-TC1, MH-TC2, MH-TC3, M2-like genes, and PI3K-related pathways. If modules (gene clusters) were detected via a dissimilarity matrix from human data, a similar set of candidate genes would be selected. Given the tuning parameters $\alpha = 0.98$ and $\lambda_{SGL} = 0.0254$, 14 genes were selected as drug-resistant biomarkers: *CCL22*, *ADCY2*, *PDK1*, *CP*, *ZFP36*, *CD2*, *PLAUR*, *ACAP1*, *COL5A1*, *FAM83D*, *PBK*, *FANCA*, *ANXA7*, and *TACC3*. Twelve of them were also selected when modules were detected using the dissimilarity matrix from mouse data. The gene names, cell types, and related pathways/gene clusters of the selected 14 genes are summarized in Table 1. Their biological functions related to gliomas are further illustrated in the discussion.

For the purpose of building a parsimonious model including a small set of genes that are most likely to serve as the potential targets for developing novel treatments, the biomarkers were further reduced by fitting L_1-penalized Cox regression model. Given the $\lambda_{\text{Lasso}} = 0.1450$, five genes were finally selected for the polygenic signature: CCL22, ADCY2, PDK1, CD2, and COL5A1. Fitting a Cox regression model on the five genes (gene expression was standardized by median and IQR) with adjustment of age, the risk score (RS) can be calculated as the linear combination of the covariates by the following formula:

$$\begin{aligned} RS = {} & 0.04203 \times \text{Age} - 0.65889 \times \text{CCL22} - 0.65991 \times \text{ADCY2} + 0.40717 \times \text{PDK1} \\ & + 1.14938 \times \text{CD2} + 0.25144 \times \text{COL5A1}. \end{aligned} \tag{11}$$

Here, CCL22 is an M2-like gene. ADCY2, PDK1, and COL5A1 are all involved in PI3K-related pathways. COL5A1 was chosen from the *MH-TC2* gene clusters and is involved in an extracellular matrix organization. CD2 was chosen from the *MH-TAM2* gene cluster associated with inflammatory response. All five of the signature genes have been demonstrated in the literature to be associated with the progression and metabolism of multiple malignant tumors, including GBM. Details are further illustrated below.

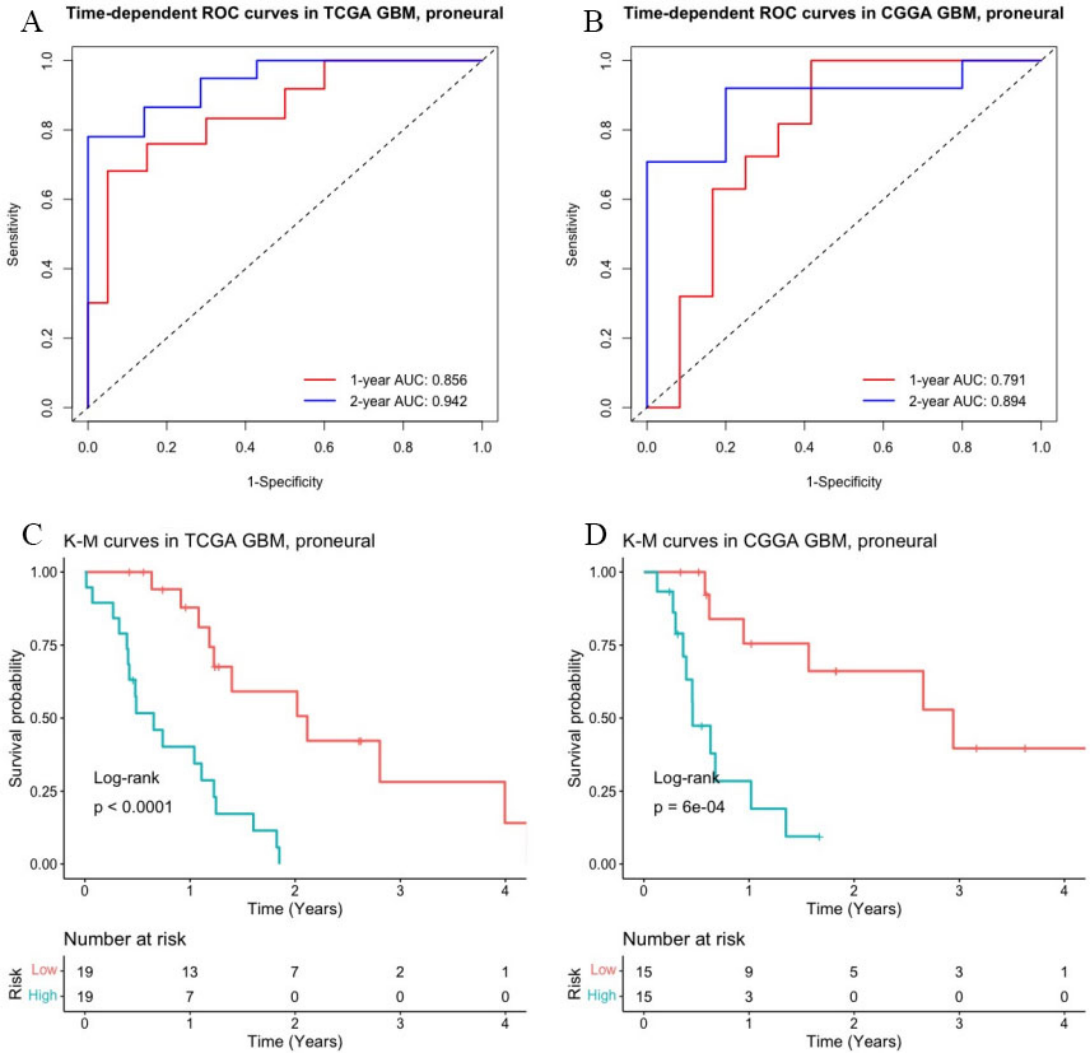

Figure 6. Evaluation of the predictive performance of the identified signature in the proneural subtype of GBM: (A) time-dependent ROC curves with corresponding AUCs at one and two years in training set (TCGA, $n = 38$); (B) time-dependent ROC curves with corresponding AUCs at one and two years in testing set (CGGA, $n = 30$); (C) KM curves for high-risk and low-risk patients in training set; and (D) KM curves for high-risk and low-risk patients in testing set. P-values were calculated from the log-rank test. TCGA: The Cancer Genome Atlas. CGGA: Chinese Glioma Genome Atlas. ROC: receiver operating characteristic. GBM: glioblastoma multiforme. KM: Kaplan-Meier.

Evaluation of the predictive accuracy of the drug-resistant signature in different subgroups

We first assessed the predictive performance of the identified signature in the proneural type GBM patients. Risk scores were calculated in both training set (TCGA) and testing set (CGGA) by Equation (12) with gene expressions standardized by median and IQR. Figure 6A shows the time-dependent ROC curves in the training set (TCGA). The corresponding 1- and 2-year AUCs were 0.856 and 0.942, respectively. Figure 6B shows the time-dependent ROC curves in testing set (CGGA). The corresponding one- and two-year AUCs in the independent testing set were 0.791 and 0.894, respectively, which demonstrated that the identified signature possessed a high predictive accuracy of survival in the proneural subtype of GBM patients. Moreover, K-M curves were generated for high-risk and low-risk patients classified by the median of the risk scores, as shown in Figure 6C and D. The overall survivals were significantly different between the high-risk group and low-risk group in both training and testing sets (Log-rank test: $P < 0.0001$ in training set and $P = 6 \times 10^{-4}$ in testing sets).

Furthermore, since many LGG will eventually progress to high-grade gliomas, with the majority of them being

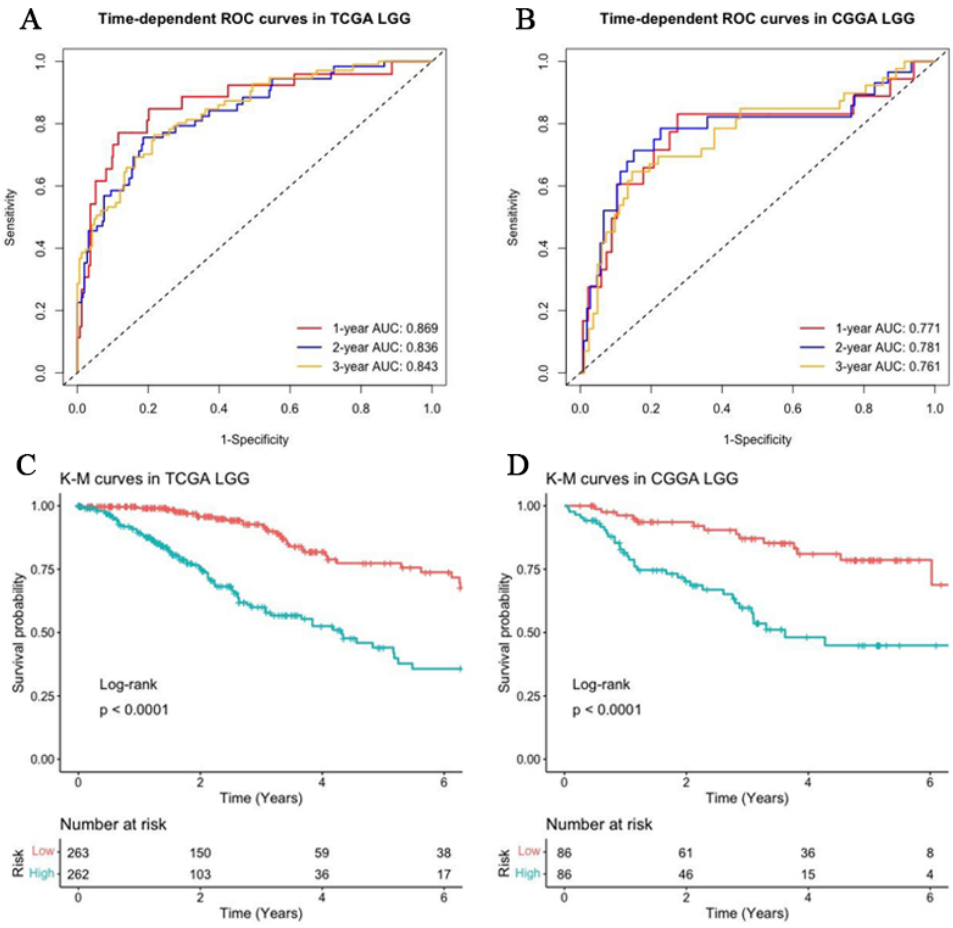

Figure 7. Evaluation of the predictive performance of the identified signature in LGG: (A) time-dependent ROC curves with corresponding AUCs at one, two, and three years in training set (TCGA, n = 525); (B) time-dependent ROC curves with corresponding AUCs at one, two, and three years in testing set (CGGA, n = 172); (C) KM curves for high-risk and low-risk patients in training set; and (D) KM curves for high-risk and low-risk patients in testing set. P-values were calculated from the log-rank test. TCGA: The Cancer Genome Atlas. CGGA: Chinese Glioma Genome Atlas. ROC: receiver operating characteristic. LGG: lower-grade gliomas. KM: Kaplan-Meier.

the proneural subtype of GBM [30,31], it would be interesting to investigate the prognostic and predictive properties of the drug-resistant signature within LGG. We therefore refitted the Cox model using the five signature genes and age in a training set of 525 LGG patients from TCGA. We validated it using an independent testing set of 172 LGG patients from CGGA. Risk scores were calculated based on the standardized expression levels. Figure 7A and B shows that the time-dependent AUC of this signature at one, two, and three years were 0.896, 0.836, 0.843 in training set and 0.771, 0.781, 0.761 in testing set. Figure 7C and D indicates that the overall survivals were significantly different between the high-risk group and low-risk group classified by the median of risk scores, as P-value < 0.0001 in both training and testing sets. These results suggest that the drug-resistant signature identified in GBM proneural subtype also has good prognostic power in LGG.

The mouse study reported by Quail *et al.* [1] was conducted on the proneural subtype of mice; thus, one may doubt whether the findings and biomarkers would be generalizable to non-proneural type of GBM patients. Indeed, in contrast to GBM proneural subtype and LGG, the identified candidate genes had very poor prognostic power in non-proneural types of GBM patients. To be specific, 127 non-proneural GBM patients collected from TCGA were used as the training set and 108 non-proneural GBM patients from CGGA were used as the testing set. We retrained the Cox model using the five candidate genes and age in the training set and calculated risk scores in both sets based on standardized expression levels. In the training set, the time-dependent

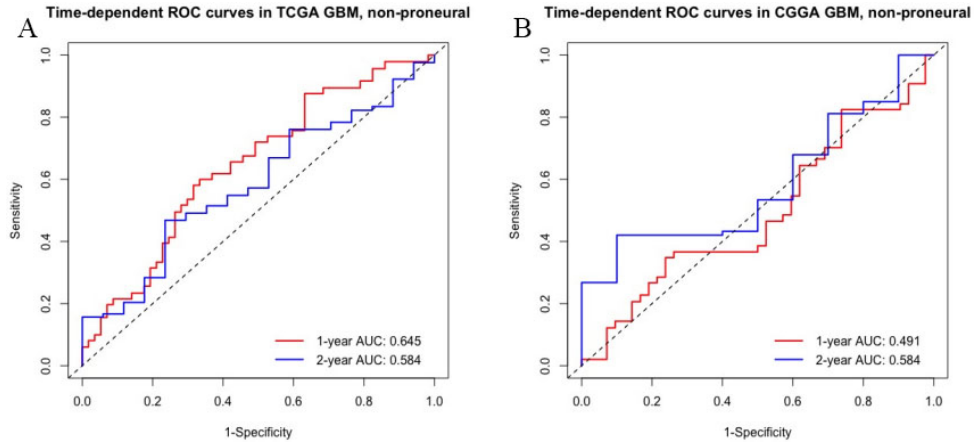

Figure 8. Evaluation of the predictive performance of the identified signature in non-proneural type of GBM: (A) time-dependent ROC curves with corresponding AUCs at one and two years in training set (TCGA, n = 127); and (B) time-dependent ROC curves with corresponding AUCs at one and two years in testing set (CGGA, n = 108). GBM: glioblastoma multiforme. TCGA: The Cancer Genome Atlas. ROC: receiver operating characteristic.

AUCs at one and two years were only 0.645 and 0.584, respectively; in the testing set, they were 0.491 and 0.584, respectively [Figure 8]. Thus, the findings from the mouse study are not generalizable to non-proneural type GBMs, which is not surprising due to different genomic profiles for the different subtypes of GBMs.

DISCUSSION

Glioma is the most malignant and invasive tumor that has a poor prognosis, with a median survival of GBM of only 12 months[12,31]. Despite the advances in cancer immunotherapy, patients still have limited sensitivity to current therapies, which implies a high prevalence of drug resistance. In fact, CSF1R inhibition as a treatment of glioma is being evaluated in some early-phase human clinical trials. However, some trials have been stopped or failed due to no evidence of survival improvement. The reported early-stage trials did not focus on subtypes of GBM, thus did not have adequate power to detect treatment effect in a subtype of GBMs given the small sample size. Even the likely responsive subgroups of GBM patients may have drug resistance[32,33]. Quail *et al.*[1] identified the drug resistance mechanism that the long-term inhibition of CSF1R in macrophage cells could activate IGF1/PI3K pathway in tumor cells and lead to drug resistance in mice. Therefore, it is of particular interest in identifying evidence to indicate whether the drug resistance mechanism in mice might also exist in human glioma patients, and whether some subgroups of GBM patients might have better survival using such therapies, in order to potentially improve the response and feasibility of this therapy in humans. In this study, we carried out a network-based, translational research strategy to identify potential targets for therapies with gene signatures that are predictive of survival and indicative of drug resistance to CSF1R inhibition treatment. Specifically, borrowing strength from the mouse study, we identified candidate genes that were differentially expressed between the drug-sensitive and drug-resistant mice, and translated those genes to human homologs. Then, those DEGs were used to construct weighted gene correlation networks in TAMs and TCs, for mice and the proneural subtype of GBM patients, respectively. Clusters of genes (modules) were detected from each of the networks, and biologically important gene clusters were identified as DEGs with top significant overlaps between human and mouse modules, incorporating results of gene-set enrichment analyses. The construction of weighted gene networks and detection of gene clusters in humans borrow information from the dissimilarity matrix from the mouse data to improve stability, given the lack of cell-specific gene expression data in humans. To obtain a smaller candidate gene set, functionally important and predictive genes were selected via cluster membership and the K-index from those gene clusters as well as M2-like and PI3K-related pathway DEGs. The regularized Cox regression models were then applied to further shrink the candidate gene set to obtain

genetic biomarkers that are more likely to be actionable, which resulted in 14 genes (*CCL22*, *ADCY2*, *PDK1*, *ZFP36*, *CP*, *CD2*, *PLAUR*, *ACAP1*, *COL5A1*, *FAM83D*, *PBK*, *FANCA*, *ANXA7*, and *TACC3*).

Knowing the selection process, it is not a surprise that all the candidate genes selected are known to play important functional roles in cancer progression, as reported in the literature. In particular, *CCL22* is an M2-like gene, while *ADCY2*, *PDK1*, and *COL5A1* belong to PI3K pathway. According to Quail *et al.*[1], resistance to CSF1R inhibition was reflected by elevated expression of M2-like genes in TAM and activation of PI3K pathway in TC. *ZFP36*, *CP*, *CD2*, *PLAUR*, and *ACAP1* were selected from the gene cluster that was enriched in inflammatory, immune response, and regulation of cytokine pathways (MH-TAM2). Inflammation and immune responses are associated with increased susceptibility to cancer development and facilitate all stages of tumorigenesis[27,34]. Cytokines are potent but complex immune mediators and have drawn great attention to the development of cancer immunotherapies[35]. *COL5A1* also came from the gene clusters that were enriched in ECM organization (MH-TC2). In tumor tissues, the growth and malignancy of the tumor as well as the response to therapy are affected by the ECM[29]. *FAM83D*, *PBK*, *FANCA*, *ANXA7*, and *TACC3* were selected from the gene cluster that was enriched in mitotic cell process pathway (MH-TC3). Aberrant activities of various cell cycle proteins can lead to uncontrolled proliferation in cancer. Targeting mitotic cell cycle has been studied as a novel cancer treatment strategy[36–38]. Since these gene clusters were identified from DEGs that were differentially expressed between the drug-resistant mice and the drug-sensitive mice, these pathways are likely to be associated with drug resistance.

In the literature, the 14 identified genes have been suggested as essential for the development and progression of many cancers including gliomas. Particularly, CCL22 (C-C motif chemokine ligand 22) is found in many types of human cancers and has lower expression levels in gliomas cases than in controls[39,40]. As a T cell trafficking chemokine, CCL22 attracts regulatory T cells (Treg), which could promote tumorigenesis. Inhibiting Treg trafficking in GBM may be a novel strategy to develop therapeutic interventions, which has been shown to be effective in other cancer models[41]. ADCY2 (adenylate cyclase 2), which is involved in the calcium signaling pathway, may play a crucial role in the development and progression of gliomas[42]. Aberrant methylation of ADCY2 is observed in many other cancers[43]. PDK1 (pyruvate dehydrogenase kinase 1) is a hypoxia-inducing factor (HIF)-1 regulated gene which may promote EGFR activation that can subsequently sustain malignant progression[44,45]. By inactivating PDK1, glioma cell colony and sphere formation could be greatly inhibited, and glioma spheres would become more sensitized to temozolomide (TMZ) toxicity[46–49]. CD2 (CD2 molecule) is a transmembrane molecule expressed on T, natural killer (NK), and dendritic cells and is essential for immunology[50,51]. It was found to be involved in tumor invasion and is highly expressed in breast cancer[50,52]. COL5A1 (collagen type V alpha 1 chain) was found to be related to the occurrence and progression of multiple types of malignant tumors, including breast cancer and gliomas. Recent studies found COL5A1 was positively correlated with the increasing malignancy of glioblastoma through the PPRC1-ESM1 axis activation and extracellular matrix remodeling, and it may be a potential therapeutic target for glioma[53–56]. FAM83D (family with sequence similarity 83 member D) is a member of FAM83 family (including FAM83A, FAM83B, and FAM83D), which has been shown to have oncogenic potential recently. FAM83D was found to be consistently upregulated across human tumor types, including gliomas[57,58]. PBK (PDZ binding kinase) expression, which is associated with cell growth and apoptosis, DNA damage repair, immune responses, *etc*., plays an essential role in tumorigenesis and metastasis. It was found to be upregulated in GBM patients[59,60]. An *in vivo* study reported that inhibition of PBK could almost completely abolish tumor growth, which made PBK serve as a potentially promising therapeutic target for GBM treatment[61,62]. FANCA (FA complementation group A) is associated with tissue proliferation and was found to be overexpressed in many types of cancers[16,63,64]. FANCA is essential for the function of Fanconi anemia (FA) pathway. Targeting the FA pathway may provide a novel strategy for the sensitization of solid tumors and investigation of chemoresistance in different tumor types[65]. ANXA7 (annexin A7) is a ubiquitinated tumor suppressor gene[66]. Loss of ANXA7 function stabilizes the EGFR protein, augments EGFR transforming signaling in glioblastoma cells,

and promotes tumorigenesis[67,68]. TACC3 (transforming acidic coiled-coil containing protein 3) is often mentioned with FGFR3-TACC3 fusion, which is an oncogenic driver. FGFR3-TACC3 fusions generate powerful oncogenes that combine growth-promoting effects with aneuploidy through the activation of as yet unclear intracellular signaling mechanisms[69,70]. FGFR inhibition has shown encouraging outcomes in mouse studies[70]. Targeting FGFR3-TACC3 fusion is evaluated by many ongoing early phase human clinical trials[70–72]. ZFP36 (ZFP36 ring finger protein) is a well-known mRNA binding protein. In the tumor microenvironment, ZFP36 might reduce the growth and invasion of glioma cells by targeting IL-13 mRNA to inhibit the role of PI3K/Akt/mTOR pathways[73–75]. CP (ceruloplasmin) serves as a prognostic biomarker in many cancers, including bile duct cancer, bladder cancer, breast cancer, *etc.*[76–78]. The expression of ACAP1 (ArfGAP with coiled-coil, ankyrin repeat and PH domains 1) is correlated with immune infiltration levels in many types of cancers[79–81]. PLAUR encodes the urokinase receptor (uPAR). The overexpression of PLAUR has been shown to be associated with poor prognosis in many types of gliomas, particularly in mesenchymal subtype GBM and LGG[82–84]. Indeed, the 14 identified genes are more likely to reflect drug resistance and serve as potential targets since they are differentially expressed between drug-resistant and drug-sensitive mice. They might be modified in patients just as they can be modified in mice, as studied by Quail *et al.*[1].

In addition, among the 14 genetic biomarkers, five genes (*CCL22*, *ADCY2*, *PDK1*, *CD2*, and *COL5A1*) were chosen to form a prognostic signature using the L_1-Cox regression model. The established signature has good prognostic power in the proneural subtype of GBM and LGG patients. We set TCGA as the training set for modeling and used CGGA, an independent cohort, as the testing set to validate the performance. In proneural subtype of GBM patients, the two-year AUC of this signature attained 0.89 in the testing set, which reveals the potential to build treatment targets for improved patient survival. Furthermore, as Quail *et al.*[1] also identified interventions to overcome the drug resistance in mice, any genetic biomarkers we identified here would likely to be modifiable targets for therapeutic intervention in humans. Thus, new clinical trials targeting proneural type GBMs might be developed. This drug-resistant signature also shows moderate time-dependent AUCs in LGG patients. Since many LGG will eventually progress to high-grade gliomas, with the majority of them being the proneural subtype of GBM[30,31], it would be interesting to investigate which LGG might progress to the proneural type of GBM, and it would be of clinical importance to find out whether a novel therapy based on our candidate target genes might prevent LGG from progression to advanced stage and prolong patient survival. Consequently, using a translational network-based multicellular analysis, we linked the drug-resistance mechanism identified in mice to population-level survival rates of both the proneural type GBM and a large number of LGG patients. Importantly, the biomarkers identified from the mouse study of proneural type have a poor predictive power of survival in non-proneural GBM patients, which implies that new biological mechanisms need to be identified for the non-proneural type of GBM patients. The 14 identified biomarkers and the signature are promising targets for therapies in glioma precision medicine, or individualized treatment, because they are potentially feasible only in some subgroups of glioma patients instead of all glioma patients.

One limitation of our human study is that we only have gene expression data from bulk tissue, not knowing expression levels in TCs and in TAMs, respectively. As the cell-specific RNA-seq gene expression data will become increasingly available in the future, one might be able to construct gene networks and models using human cell-specific RNA-seq data, which would further improve the efficiency and precision of the methods we developed here to identify candidate biomarkers. In addition, with cell-specific human gene expression data on TAMs and on TCs, we would be able to model and investigate the interactions between TAMs and TCs, which is clearly important in directly investigating the mechanisms of drug resistance in humans and the identification of novel treatment targets to overcome drug resistance and prolong survival of patients.

DECLARATIONS

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Authors' contributions

Conceptualization, investigation, writing: Lu Y
Conceptualization, supervision, writing: Shao Y

Availability of data and materials

The mouse RNA-seq gene expression data is available on Gene Expression Omnibus (GEO) website (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE69104. TCGA glioma data can be downloaded from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/). CGGA glioma data can be downloaded from Chinese Glioma Genome Atlas (CGGA) database (http://www.cgga.org.cn/).

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

There is no ethical issue in studying de-identified published publicly available data. IRB approval was waived.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Editorial

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Emerging insights to lung cancer drug resistance

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Abstract

Lung cancer remains the malignant tumor with the highest morbidity and mortality in China, with non-small cell lung cancer (NSCLC) accounting for 80%-85% of cases. Nowadays, the treatment pattern of NSCLC has evolved toward precision management with the development of molecular targeted therapy and immunotherapy. However, the median overall survival for patients with metastatic NSCLC, unfortunately, remains less than three years. Drug resistance is the bottleneck to preventing drugs from playing a further role, and the mechanistic study of drug resistance is the prerequisite for new regimen development. This Special Issue pays special attention to drug resistance in the treatment of NSCLC. We received and published several excellent articles regarding this topic. We hope that, through this Special Issue, we can have a deep understanding of the existing problems, the underlying mechanism, and the future solutions and that the publication of this Special Issue can bring some inspiration to readers.

Keywords: Non-small cell lung cancer, drug resistance, targeted therapy, immunotherapy

INTRODUCTION

According to the most recent statistics from the National Cancer Center of China[1], lung cancer remains the most common malignant tumor as well as the first leading cause of cancer-related deaths in China, with an estimated 0.828 million new cases and 0.657 million deaths per year, placing a heavy burden to public health

and economy development. Approximately 80%-85% of lung cancers are classified pathologically as non-small cell lung cancer (NSCLC)[2]. With substantial improvements in understanding of tumor biology, genomic mutational landscapes, cancer immunology, and tumor microenvironment, the treatment pattern of NSCLC has evolved toward precision management[3]. Molecular targeted therapy based on different driver oncogenes and immune checkpoint inhibitors (ICIs) based on programmed cell death ligand 1(PD-L1) expression are the 2 major branches of personalized treatment paradigm, which have brought great survival benefits to patients with oncogene-addicted and non-oncogene-addicted cancers, respectively[4]. However, the median overall survival for those presenting with metastatic NSCLC, unfortunately, remains less than 3 years[4]. Drug resistance is always the bottleneck to preventing drugs or new regimens from playing a further role. This Special Issue pays special attention to drug resistance in the treatment of NSCLC. We hope that, through this issue, we can have a deep understanding of the existing problems, the underlying mechanism, and the future solutions and that the publication of this Special Issue can bring some inspiration to readers.

MAIN TEXT

Resistance mechanism for targeted therapy

EGFR

The activating mutation of epidermal growth factor receptor (EGFR) is highly common in Asian patients with NSCLC, accounting for 40%-50% of cases[5]. Despite the significant benefits of EGFR-tyrosine kinase inhibitors (EGFR-TKIs), patients with EGFR activating mutation inevitably develop drug resistance within a certain treatment period. Nowadays, many studies focus on resistance mechanisms to third-generation EGFR-TKIs, as they have been recommended as the preferred first-line treatment for advanced NSCLC patients with EGFR activating mutation. Numerous resistance mechanisms have been identified[6-8], which can be broadly classified into two groups: (1) The first includes EGFR-dependent mechanisms, mainly referring to secondary or tertiary mutations, such as C797S, G724, L792, L718, and G719; (2) the second includes EGFR-independent mechanisms, including activation of downstream signaling pathways such as human epidermal growth factor receptor 3 (HER3), anexelekto (AXL), fibroblast growth factor receptor (FGFR) signaling pathways, *etc.*; acquisition of other potentially targetable oncogenic drivers such as mutations in phosphatidylinositol-3-kinase catalytic α (PIK3CA), Kirsten rat sarcoma (KRAS), BRAF V600E, anaplastic lymphoma kinase (ALK) fusions, MET amplification, *etc.*; and histological transformation to small-cell or squamous carcinoma.

Recently, some new mechanisms have been revealed. For example, Kashima *et al.* identified CD74 upregulation as a novel mechanism of resistance to osimertinib by applying single-cell analyses to cell models[9]. Nilsson *et al.* found that the activation of yes-associated protein (YAP) and forkhead box protein M1 (FOXM1) could mediate epithelial-to-mesenchymal transition (EMT)-associated EGFR-TKIs resistance[10]. These mechanistic studies have promoted some potential strategies to overcome EGFR-TKIs resistance. The allosteric inhibitor of src homology 2 domain-containing phosphatases (SHP2), a blockade to receptor tyrosine kinases (RTK) signaling, was recently shown to be a therapeutic strategy in acquired EGFR-TKIs-resistant NSCLC at the preclinical stage[11]. The combination of osimertinib and a novel AXL inhibitor ONO-7475 showed remarkable activity on osimertinib-resistant tumor cells *in vitro* and *in vivo*[12]. Zhu *et al.* identified a novel connection between osimertinib and c-Myc and further demonstrated targeting c-Myc as a potential strategy to overcome osimertinib acquired resistance in cell lines[13]. However, these therapeutic strategies need to be further validated in clinical trials and more research is welcomed.

KRAS

The KRAS gene is the most frequently mutated oncogene in human cancer, accounting for approximately 30% of NSCLC[14]. However, because RAS protein has a picomolar affinity for GTP and lacks known allosteric regulatory sites, KRAS had long been considered "undruggable" despite 40 years of sustained efforts. In 2013, the breakthrough by Ostrem *et al.* of small molecules that covalently bind to the acquired cysteine residue within the switch II region in $KRAS^{G12C}$ opened the door to therapy targeting KRAS[15]. In recent years, a series of clinical trials have evidenced the remarkable antitumor activity of $KRAS^{G12C}$ inhibitors such as AMG510 and MRTX849, with a response rate of 30%-40%[16,17]. However, drug resistance also has limited survival benefits for most patients, with a median progression-free survival of 6.3 months. Similarly, the resistance mechanisms of $KRAS^{G12C}$ inhibitors are divided into the KRAS-dependent and -independent ways. The reported acquired KRAS alterations[18,19] include G12D/R/V/W, G13D, Q61H, R68S, H95D/Q/R, Y96C, Y96D, Y96S, and high-level amplification of the $KRAS^{G12C}$ allele, whereas KRAS-independent mechanisms[20,21] also involve bypass signaling activation, such as upstream RTK regulators (EGFR, HER2, FGFR, and SHP2), direct mediators of KRAS activation (AURKA), and/or effectors of mitogen-activated protein kinase (MAPK) and PI3K pathways (MYC and mTOR); acquired alterations of other oncogenes, such as NRAS, BRAF, MAP2K1, RET, ALK, FGFR3, CDKN2A, PTEN, *etc.*; and histological transformation to squamous cell carcinoma. In addition, impaired antitumor immunity could confer resistance to $KRAS^{G12C}$ inhibition independent of the above mechanisms, which might be attributed to the partial antitumor efficacy of $KRAS^{G12C}$ inhibitors derived from the activation of an immune response, as $KRAS^{G12C}$ inhibition could induce a pro-inflammatory tumor microenvironment (TME) and prime antigen-presenting cells and cytotoxic T cells[22]. Therefore, the feasibility and efficacy of the combination of $KRAS^{G12C}$ inhibitor with immunotherapy are worthy of further studies.

Together, although drugs targeting KRAS were first developed less than 10 years ago and only two kinds of targeted drugs have entered the clinic and are both under phase III clinical trials now, the mechanisms of drug resistance have been studied to develop effective therapeutic strategies better. However, more relevant clinical data are needed in the near future when the drugs are widely used in clinical practice.

Resistance mechanism for ICIs-based therapy

Immune checkpoint inhibitors (ICIs) targeting programmed cell death 1 (PD-1) and its ligand (PD-L1) have revolutionized the treatment scenario for patients with NSCLC. Albeit with unprecedented improved long-term survival in selected patients, ICIs failed to achieve a high response rate for the whole population. Because of the specific kinetics and patterns of response to ICIs, their resistance mechanisms are much different from those of chemotherapy or targeted therapy. In 2020, the Society for Immunotherapy of Cancer published an expert consensus on clinical definitions for resistance to ICIs in three distinct scenarios: primary resistance, secondary resistance, and progression after treatment discontinuation[23].

Primary resistance is defined as evidence of disease progression after receiving at least 6 weeks (2 cycles) of exposure to ICIs but no more than 6 months. This type of resistance is related to the inability of the immune system to activate an appropriate immune response to malignant neoplasm, which can be due to any impaired functions in the cancer-immunity cycle[24], including cancer antigen release and presentation; T-cell priming, activation, trafficking, migration, and infiltration; or recognition and killing of cancer cells by T cells. Factors contributing to this resistance involve tumor-intrinsic and tumor-extrinsic mechanisms. Tumor-intrinsic mechanisms refer to the cancer cells enabling the suppression of the immune response by modulating their genome, transcriptome, and proteome. Some gene alterations[25,26] [loss of phosphatase and tensin homolog (PTEN), the mutation in Liver kinase B1 (LKB1), TP53, EGFR, ALK, *etc.*], and oncogenic signaling pathways[27] (WNT/β-catenin, MYC, MAPK, JAK/STAT3, *etc.*) decrease T-cell infiltration. LKB1-mutation also results in the recruitment of suppressive myeloid cells, and the inhibition of granulocytic

myeloid-derived suppressor cells (G-MDSCs) via all-trans-retinoic acid could overcome the resistance of PD-1 blockade in LKB1-deficient murine NSCLC[28]. Yang *et al.* discovered that USP12 downregulation fostered an immunosuppressive microenvironment with increased macrophage recruitment and reduced T-cell activation via NF-κB hyperactivation in tumor cells[29]. Recently, Wennerberg *et al.* described the expression of mono-adenosine 5'-diphosphate-ribosyltransferase 1 (ART1) on tumor cell-mediated cell death of P2X7R+ CD8 T cells as a novel mechanism of immune resistance in NSCLC and provided preclinical evidence that antibody-mediated targeting of ART1 could improve tumor control[30]. Tumor-extrinsic factors are those components beyond cancer cells that contribute to suppressive TME. For example, the overexpression of vascular endothelial growth factor (VEGF) on endothelial cells promotes MDSC infiltration and decreases T-cell infiltration[31]. Cancer-associated fibroblasts (CAF), one of the most abundant components of TME, could also create an immune-suppressive TME by increasing the infiltration of MDSCs and tumor-associated macrophages, promoting the polarization of macrophages and reducing proliferation and antitumor activity of $CD8^+$ T cells and natural killer cells[32]. Recently, Horton *et al.* reported that abnormal differentiation of $CD8^+$ T cell during priming mediated ICIs resistance in T cell-infiltrated NSCLC[33].

Acquired resistance is defined as evidence of disease progression after experiencing clinical benefit (either objective response or stable disease lasting 6 months or greater). As the name implies, this type of resistance is "acquired" through the adaption of tumor cells to the host immune system, reflecting the evolution of the tumor as a malignant organism under selection pressure from therapeutic drugs. Early research[34] suggested that tumor cell-autonomous defects in interferon (IFN) signaling through JAK1/2 inactivating mutations or HLA class I antigen processing through mutation or loss of beta-2-microglobulin (B2M) mediated acquired resistance to ICIs. Neoantigen depletion was also identified to lead to subsequent immune evasion in NSCLC[35]. The upregulation of additional coinhibitory receptors[36], such as LAG-3, TIM-3, TIGIT, VISTA, *etc*., could also abrogate the effect of PD-1/PD-L1 blockade. In addition, acquired resistance to ICIs could also arise upon selection for new oncogenic variants that mediate T-cell exclusion, including loss of PTEN and WNT/β-catenin activation[34]. Recently, the upregulation of CD38 induced by ATRA and IFN-β was found to mediate acquired resistance by suppressing cytotoxic T-cell proliferation, antitumor cytokine secretion, and killing capability via adenosine receptor signaling[37].

Considering the special context that disease progression occurs after discontinuation for adjuvant/neoadjuvant immunotherapy with a fixed duration, or for the metastatic setting either secondary to toxicity or after achieving maximal benefits, the conception of "progression after treatment discontinuation" was proposed. In terms of mechanism, this type of resistance can be mediated by the elements from either "primary resistance" or "acquired resistance". The early recurrence after discontinuation of adjuvant therapy may resemble primary resistance, whereas late recurrence after adjuvant therapy discontinuation and initial disease control may resemble acquired resistance. However, up to now, studies focused on resistance mechanisms specifically in these settings are lacking.

Taken together, resistance mechanisms to ICIs are much different from those to chemotherapy or targeted therapy, in which tumor-intrinsic and tumor-extrinsic factors both need to be considered. Although there are many barriers to investigating resistance mechanisms to immunotherapy, including difficulty acquiring optimal tumor samples for analyses and the absence of routine and effective tools to comprehensively interrogate alterations in the tumor, host, and/or microenvironment, more in-depth studies are needed to better clarify the underlying molecular mechanism and develop corresponding rational therapeutic strategies to overcome resistance. Of note, there is a lack of studies on resistance mechanisms to combinations of ICIs and chemotherapy, since the synergistic antitumor activity leaves it difficult to

distinguish which component is the driver of response.

In this Special Issue, some new factors are reported to contribute to ICI resistance. Wu *et al.* found that circRNA hsa_circ_0020714 was related to a poor prognosis of anti-PD-1 immunotherapy in NSCLC, and a mechanistic study suggested that hsa_circ_0020714 functioned as an endogenous miR-30a-5p sponge to enhance SOX4 expression, thereby promoting immune evasion and anti-PD-1 resistance in NSCLC patients[38]. Their study raised the important role of circRNA in immune resistance and provided a potential targeted pathway "hsa_circ_0020714/miR-30a-5p/SOX4" to overcome resistance to anti-PD-1 therapy. The role of tumor-derived exosomes in tumor response to immunotherapy was recapitulated by Wu *et al.*, and they pointed out that tumor-derived exosomes should be studied and manipulated to provide clinical benefits and improve the clinical management of lung cancer[39]. Li *et al.* reviewed the resistance mechanism to ICIs, especially in KRAS-mutant NSCLC, with a critical focus on metabolism remodeling mediated by the oncogenic KRAS pathway, and they argued that these alternated metabolic pathways could be promising approaches to overcome immunotherapy resistance[40]. From the present clinical practice, Yu *et al.* discovered that the use of ICIs plus chemotherapy and/or anti-angiogenesis therapy correlated with better survival after resistance to previous ICI treatment, which provides a therapeutic option for patients with resistance to ICIs before more reliable strategies enter the clinic[41]. More research on lung cancer drug resistance is needed to improve patient survival in the future.

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Authors' contributions

The author contributed solely to the article.

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Cancer Drug Resistance

Review

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Liquid biopsies in primary and secondary bone cancers

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Abstract

Liquid biopsies are a powerful tool to non-invasively analyze tumor phenotype and progression as well as drug resistance. In the bone oncology field, liquid biopsies would be particularly important to develop, since standard biopsies can be very painful, dangerous (e.g., when found in proximity to the spinal cord), and hard to collect. In this review, we explore the recent advances in liquid biopsies in both primary (osteosarcoma and Ewing sarcoma) and secondary bone cancers (breast, prostate, and lung cancer-induced bone metastases), presenting their current role and highlighting their unexpressed potential, as well as the barriers limiting their possible adoption, including costs, scalability, reproducibility, and isolation methods. We discuss the use of circulating tumor cells, cell-free circulating tumor DNA, and extracellular vesicles for the purpose of improving diagnosis, prognosis, evaluation of therapy resistance, and driving therapy decisions in both primary and secondary bone malignancies.

Keywords: Liquid biopsy, bone metastasis, osteosarcoma, Ewing sarcoma, extracellular vesicles, minimal residual disease, drug resistance

INTRODUCTION

Normal bone physiology: cellular players and the "virtuous cycle"

Bone is a connective tissue composed of a mineralized matrix and a cellular component. The former is

made up of an inorganic part (mainly calcium phosphate) and an organic one, composed of type I collagen fibers as well as a plethora of proteins such as osteonectin, osteopontin, osteocalcin and proteoglycans. This bone matrix is also a storage for several growth factors belonging to the transforming growth factor (TGF)/insulin-like growth factor (IGF) superfamilies[1,2]. The two matrix components make bone a composite material with outstanding mechanical properties, but this comes with a caveat: bone needs to be modeled and remodeled during growth and adult life, respectively, otherwise its mechanical properties degrade over time, as is the case for elderly individuals of both genders and for some bone genetic diseases[3,4]. Proper bone modeling/remodeling is guaranteed by a finely tuned balance among the three main bone cells: osteoblasts, the bone-forming cells; osteoclasts, the bone-resorbing cells; and osteocytes, key controllers of bone matrix homeostasis[5,6]. During bone modeling, all bone sections change shape following both mechanical cues and microenvironmental factors and expand to accommodate the growing organism in physiological conditions. In adults, bone switches to remodeling, where osteoblasts and osteoclasts act on the same surfaces and constantly resorb and depose new bone, resulting in a net bone mass balance, following physiological mechanical stimuli as well as the organismal needs for calcium and phosphate[7]. Although this might appear to be a waste of energy, it is crucial to maintain proper mechanical features and guarantee the repair of microfractures that are detected by osteocytes, which subsequently drive the remodeling of the damaged surfaces. Osteocytes are also able to sense mechanical loading, which acts as an anabolic stimulus that promotes osteoblast activity[8,9]. Since the process of bone resorption-deposition in homeostatic conditions keeps bone at its best, and it is cyclic, bone researchers often use the term "virtuous cycle" to describe it.

Primary bone tumors

Osteosarcoma is the most common primary bone tumor. It has two separate incidence peaks, the highest one between 10 and 20 years of age and a secondary one in people aged ≥ 65 years [10]. Although the overall incidence of this cancer is low (3.1 cases per million per year in the US)[11], it represents 2% of all cancers in 0-14-year-old and 3% in 14-19-year-old[12], and its consequences can be devastating, ranging from limb amputation (20% of operable osteosarcomas) to death because of lung metastases[11]. Osteosarcoma derives from a malignant transformation of the mesenchymal cells that can present different cellular features, but they all have in common the deposition of osteoid-like matrix and mineralized or demineralized lesions that are clearly visible upon X-ray examination[13]. When the gold standard treatment, i.e., neo-adjuvant chemotherapy-surgery-adjuvant chemotherapy, is not possible, the only available treatment course is chemotherapy alone, since radiotherapy does not provide significant benefits and increases the risk of infection[14]. However, despite advances in the field, chemoresistance is a serious problem in this type of neoplasia, and the survival benefits of chemotherapy are limited due to the onset of drug resistance[14-16], which eventually leads to lung metastasis development, inevitably ending with the death of the patient. Chemotherapy is therefore a limited tool in osteosarcoma management, but having a reliable way to monitor the onset of drug resistance would make it much more effective.

There is still no consensus on the cell from which Ewing sarcoma originates, but what is certain is that it most often localizes in bone (80%-85%) or the soft tissue surrounding it (15%-20%)[17]. Ewing sarcoma also has quite a high incidence of 2.93 per million in newborns to adolescents[18]. Moreover, 85% of Ewing sarcomas harbor a specific chromosomal translocation [t(11:22)(q24:q12)] leading to the fusion of the N-terminal portion of Ewing sarcoma gene (EWS) with the C-terminal portion of Friend leukemia integration 1 transcription factor (FLI1), which encodes for a chimeric protein that behaves as a transcription factor and modulates a plethora of cellular processes, eventually leading to malignant transformation[17]. While most other bone cancers have specific morphological features that make tracking their lineage relatively easy, Ewing sarcoma cells are round, small basophils and defined as "uniformly undifferentiated"[17]. Despite advances in the field of Ewing sarcoma treatment, 70% of metastatic patients, in whom lungs are most

frequently affected as a secondary site, will eventually perish[19]. Treatment of this tumor is multimodal and comprises surgery, radiotherapy, and chemotherapy[20]. However, drug resistance is a very concrete issue, which sadly tends to increase after more aggressive rounds of chemotherapy that, in fact, do not seem to provide significant survival benefits and is especially marked in relapsing Ewing sarcoma[21,22]. Hence, monitoring the onset of drug resistance with a non-invasive technique such as liquid biopsy could be a valuable tool in the fight against this family of tumors as well.

Bone metastases and the "vicious cycle"

The bone milieu is a particularly attractive microenvironment for metastasis development by many primary tumors, such as breast, prostate, lung, and kidney cancers[23-25]. There could be several reasons for this "preference", such as the richness in growth factors of the bone matrix[26], the presence of higher levels of calcium, which acts as a growth-promoting factor[27,28] especially following bone resorption, and the particular structure of bone/bone marrow blood vessels, which are fenestrated, hence quite permissive for tumor cells extravasation. In fact, metastasis is a stepwise process, in which first cancer cells have to invade locally in the primary site, after gaining the expression of specific sets of molecules such as matrix metalloproteinases[29,30], migrate into the bloodstream or lymphatic system, escape immune killing while circulating, extravasate in the secondary site, engraft, and finally survive in the secondary organ. The final parts of the process, spanning from the extravasation to the survival in the new microenvironment, are often termed "homing" and require cancer cells to "trick" the resident cells into believing that they actually belong there, expressing bone-specific factors (osteomimicry)[31] or hematopoietic stem cell-specific factors (HSC-mimicry)[32].

Depending on the type of cancer, osteoblasts, osteoclasts, and osteocytes are affected in specific ways. In the case of breast cancer, metastatic dissemination usually causes the local degradation of the bone matrix, which is evident on X-ray analysis as void areas where bone should be. These types of metastases are termed "osteolytic", and while both osteoblasts and osteoclasts are involved in their establishment, the main culprit for their radiographic appearance and fueling are osteoclasts, which become overactivated following their interaction with cancer cells[33,34]. On the other hand, prostate cancer preferentially causes a net activation of osteoblasts in the bone metastatic microenvironment, leading to abnormally high bone deposition and the onset of radio-dense spots identifiable by X-ray examination. These metastases are termed "osteosclerotic" or "osteoblastic"[24]. Both cancers can present osteolytic and osteosclerotic features in the same anatomical site, and in this case, bone metastases are defined as "mixed"[23]. The process leading to osteolytic or osteosclerotic lesions relies on a pathological cross-communication between cancer cells and bone cells, where the former tilt the balance towards bone resorption or bone deposition, hijacking the virtuous cycle of cross-regulation between osteoblasts and osteoclasts into an osteolytic or osteosclerotic "vicious cycle".

In the former, cancer cells that reached the bone/bone marrow microenvironment secrete factors that induce a net increase in osteoclast differentiation and activity. These factors can act directly [e.g., tumor necrosis factor-α, interleukin (IL)-6, and IL-1β], and/or indirectly [like parathyroid hormone-related protein (PTHrP)], through the promotion of osteoblastic expression of pro-osteoclastogenic factors such as receptor activator of nuclear factor κB (RANKL) and macrophage colony-stimulating factor (M-CSF)[26,33-35]. The increase in osteoclast activity and differentiation leads to the degradation of bone matrix and the release of IGF-1, TGF-β, platelet-derived growth factor (PDGF), and bone morphogenetic proteins (BMPs) from it, which in turn stimulate tumor growth, thus creating a feed-forward loop, with bone cells, i.e., the osteolytic vicious cycle. In the osteosclerotic vicious cycle, cancer cells secrete a different set of proteins, including IGF-1, wingless-related integration site-1 (WNT-1) and WNT-3A, BMPs, and endothelin (ET)-1. These promote osteoblast differentiation and activity, leading to deposition of primary bone and production of growth factors[24,36,37]. Tumor growth is therefore fomented by osteoblasts, thus

closing the osteosclerotic vicious cycle. Osteoblast differentiation also stimulates osteoclast differentiation, which further exacerbates the release of matrix-bound growth factors, with dangerous and painful consequences[23,37].

LIQUID BIOPSY IN BONE ONCOLOGY

Liquid biopsies: what, how and why

Liquid biopsies are obtained in body fluids that are in direct contact with cancerous tissue and can provide important insights into the tumor without having to remove a part of it directly[38,39]. This is especially important for those tumors that are not easily accessible and for which even drawing a simple biopsy core may be dangerous and/or extremely painful. Furthermore, classical biopsies may be taken from a relatively small area of the tumor and may not be representative of it as a whole[40]. Although depending on the neoplasia might be useful to use urine, cerebrospinal fluid, and even saliva as a liquid biopsy, the body fluid that is most useful for this application is blood[39]. An additional advantage of using liquid biopsies from blood is that they can be multiplexed and implemented with classical as well as next-generation cancer profiling analyses, including circulating bone turnover biomarkers such as carboxy-terminal telopeptide cross-linked type 1 collagen, pro-peptide of type 1 collagen, bone sialoprotein, tartrate-resistant acid phosphatase 5B (TRAcP5B), and osteoprotegerin[41,42]; metabolites such as pyridinoline and deoxypyridinoline[43]; and protein markers correlating with poor outcome in bone cancers, including vascular endothelial growth factor (VEGF)[44], metallothionein[45], IL-4, and IL-8[46,47]. There are three main types of actionable biological materials that can be obtained from a blood liquid biopsy: (i) circulating tumor cells (CTCs); (ii) cell-free circulating tumor DNA (ctDNA); and (iii) extracellular vesicles (EVs, also referred to as exosomes)[48].

CTCs

As the name suggests, CTCs are tumor cells that extravasate into the blood flow. They were first identified by Ashworth more than 150 years ago and are now commonly used in clinical practice[49]. Although isolating CTCs has historically been a challenge because of their low number in the general circulation, newly developed microfluidic platforms (e.g., the FDA-approved CellSearch platform) are making this task easier, and next-generation sequencing approaches made it possible to deeply phenotype every single circulating tumor cell isolated[50]. These important advances mean that a simple liquid biopsy can provide information about the genetic mosaicism of the primary tumor, their mutational landscape, epigenetics, and even their gene and protein expression.

Circulating tumor (ct)DNA

Cell-free ctDNA arises from apoptotic and necrotic primary tumor cells, which release their cellular content into the general circulation, as demonstrated by the fact that most of the ctDNA detected is 180-200 bp in length, which is consistent with what is observed in apoptotic cells[48]. Circulating tumor DNA can provide important information about tumor mutations and copy number variation and suggests whether to proceed with a specific-target therapy or not. For example, the V600E mutation in v-raf murine sarcoma viral oncogene homolog B1 (BRAF) is found in several tumors, including metastatic colorectal cancer[51], melanoma[52,53], papillary thyroid cancer[54], etc. The V600E variant of BRAF is targetable with drugs such as vemurafenib, dabrafenib, and trametinib, and its presence can drive the choice of therapy[55].

Extracellular vesicles

The third class of biological material that is usable as liquid biopsy includes extracellular vesicles. These are lipid bilayer particles ranging from 30 to 1000 nm in diameter, which, according to their size, can be classified into three main types: apoptotic bodies, large extracellular vesicles (also known as microvesicles), and small EVs (also known as exosomes). These also differ in biogenesis and biological function[56,57].

Importantly, EVs secreted by cells often mimic the molecular composition of the cell of origin, and they contain DNA, miRNAs, mRNAs, proteins, and other biological molecules that are interesting for theranostic and prognostic purposes[56,58]. It has been thoroughly demonstrated that EVs have an important role in cancer[59,60], including bone metastases[60-63], osteosarcoma[64-66], and Ewing sarcoma[67,68], and cancer cells secrete more EVs than their normal counterparts[69]. Moreover, hypoxia, higher intracellular calcium or lower pH, oxidative stress, ionizing radiation, and ultrasounds have all been shown to increase EV production in both normal and cancer cells[70].

EVs are especially interesting for studying RNAs because these molecules are prone to be degraded in the circulation; however, when they are encapsulated into EVs, they become more stable and can be analyzed to gain insights into the transcriptional profile of the cells of origin, as well as regarding miRNAs and long non-coding RNAs[71]. A summary of the primary and secondary tumors and the potential information that can be gained by liquid biopsies is schematized in Figure 1.

Adopting different approaches to acquire all this biological information from a patient's blood could be possible to perform accurate diagnosis, estimate prognosis, and even monitor the onset of drug-resistant clones to make informed therapeutic choices and change therapy during treatment[72].

Despite the advances in the field at the preclinical level, clinical adoption of bone liquid biopsies remains poor due to costs, scalability, reproducibility, and isolation methods. In the following sections, we focus on the recent developments, as well as preclinical and clinical applications of liquid biopsies in bone oncology, focusing on both secondary [Table 1] and primary [Table 2] tumors.

Liquid biopsy in bone metastasis

All the key components in liquid biopsies, i.e., ctDNA, CTCs, and EVs, have been exploited to monitor bone metastases secondary to breast, prostate, and lung cancers at the preclinical level.

ctDNA for bone metastasis detection

Circulating tumor DNA analysis may provide a complete genetic profile of the mutational landscape of metastatic disease, and is also correlated with patients' relapse or changes in response to surgical or pharmacological treatment[73]. In this regard, a retrospective study of patients with primary breast cancer showed that the detection of metastatic disease, also spread in the bone, was possible by serial measurements of selected tumor-specific chromosomal rearrangements in ctDNA using droplet-based digital PCR technologies from plasma samples, with an average of almost one year before clinical recurrence detection during the follow-up of the disease, and the ctDNA amount was directly proportional to poor survival[74]. This highlights the possibility of using ctDNA detection as a diagnostic tool for earlier prediction of metastasis. Liquid biopsies also hold the potential to detect minimal residual disease (MRD), thus providing indications for therapy and prognosis. Detection and analysis of plasma tumor-associated ctDNA were found to be a good indicator of MRD identification and monitoring in breast cancer patients with a high risk of recurrence[75,76]. In particular, the detection of ctDNA at baseline was associated with a higher incidence of bone metastasis and subsequent poor prognosis in newly diagnosed patients with advanced non-small cell lung cancer (NSCLC)[77]. Consistently, quantification and analysis of ctDNA in late-stage NSCLC patients revealed that higher ctDNA levels were detected in the group of patients with bone metastasis[78]. It should be noted that using liquid biopsies as a means of detecting MRD is still a developing field, and the risk of false positives and false negatives is a concrete one that needs to be addressed in larger-scale longitudinal studies. Regarding the use of ctDNA, to the best of our knowledge, there are no studies focusing specifically on prostate cancer bone metastases. However, Vandekerkhove *et al.* showed that

Table 1. Selected studies about liquid biopsy applications in bone metastatic tumors

Tumor type	Source material	Target	Utility/use	References
Breast cancer	ctDNA	Tumor chromosomal rearrangements, *ESR1* mutations	Early diagnosis of BM	[74,92]
	ctDNA	*TP53*, *PIK3CA*, *ESR1* mutations	Prognosis and treatment efficacy	[89,93]
	ctDNA	*PIK3CA* mutations	Diagnosis of BM	[90]
	ctDNA	Somatic genomic alterations (*PIK3CA* and *TP53*)	Prognosis and treatment efficacy	[91]
	CTCs	Baseline CTC/mL of blood (≥ 5 CTC/7.5 mL)	Diagnosis and prognosis of BM	[80,81,89]
	EVs	Upregulation of *SPP1*, *HSP90AA1*, *IL3*, *VEGFA*, *PTK2* and *YWHAZ* genes	Early detection of BM	[94]
Lung cancer (NSCLC)	ctDNA	*KRAS*, *EGFR*, *BRAF* mutations	Early diagnosis of BM	[77]
	ctDNA	*KRAS* and *EGFR* mutations	Diagnosis and prognosis of BM	[78]
	CTCs	CTCs/mL of blood (≥ 5 CTC/7.5 mL)	Diagnosis and prognosis of BM	[84,85]
	EVs/cmiRNAs	hsa-miR-574-5p, hsa-miR-328-3p, hsa-miR-423-3p	Early detection and monitoring of BM	[95]
Prostate cancer	ctDNA	*TP53* mutations and DNA repair defects	Diagnosis of BM	[79]
	CTCs	CTCs/mL of blood (≥ 5 CTC/7.5 mL)	Diagnosis and prognosis of BM	[86]
	EVs/cmiRNAs	miR-181a-5p	Diagnosis of BM	[96]

NSCLC: Non-small cell lung cancer; ctDNA: cell-free circulating tumor DNA; CTCs: circulating tumor cells; EVs: extracellular vesicles; cmiRNAs: circulating miRNAs; BM: bone metastasis; ESR1: estrogen receptor 1; TP53: tumor protein 53; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SPP1: secreted phosphoprotein 1; HSP90AA1: heat shock protein 90 alpha family class A member 1; IL3: interleukin-3, VEGFA: vascular endothelial growth factor A; PTK2: protein tyrosine kinase 2; YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein; BRAF: B-Raf proto-oncogene; serine/threonine kinase; KRAS: KRAS proto-oncogene; EGFR: epidermal growth factor receptor.

Table 2. Selected liquid biopsy applications in primary bone tumors

Tumor type	Source material	Target	Utility/use	References
Osteosarcoma	ctDNA	Somatic mutations	Diagnosis and prognosis	[100]
	ctDNA	Chromosome arm 8q copy number gains	Genotyping and diagnosis	[101]
	CTCs	Baseline CTC/mL of blood (≥ 5 CTC/7.5 mL)	Diagnosis, prognosis, response to therapy	[104,105]
	CTCs	CTC count variations after therapy	Prognosis and treatment efficacy	[106,107]
	EVs	Transcriptomic alterations	Diagnosis of BM	[111]
	EVs/cmiRNAs	miR-148a, -574-3p, -214, -335-5p, -491, -221, -191, -421, -124, -101 and -195	Diagnosis and prognosis	[112]
Ewing sarcoma	ctDNA	*STAG2* and *TP53* mutations, *EWSR1-FLI1* and *EWSR1-ERG* fusion genes	Diagnosis, prognosis and response to therapy	[102,103]
	CTCs	CTCs count, CD99 expression and chromosomal translocations (EWSR1- FL11 fusion gene)	Diagnosis and prognosis of metastasis	[108]
	EVs/cmiRNAs	miR-125b	Diagnosis, prognosis and response to therapy	[121]
	EVs	Proteomic content (CD99, HINT1 and NGFR)	Diagnosis and prognosis	[122]

CtDNA: Cell-free circulating tumor DNA; CTCs: circulating tumor cells; EVs: extracellular vesicles; cmiRNAs: circulating miRNAs; STAG2: stromal antigen 2; TP53: tumor protein 53; EWSR1: Ewing sarcoma RNA binding protein 1; FLI1: friend leukemia integration 1 transcription factor; ERG: erythroblast transformation specific-related gene; HINT1: histidine triad nucleotide-binding protein 1; NGFR: nerve growth factor receptor.

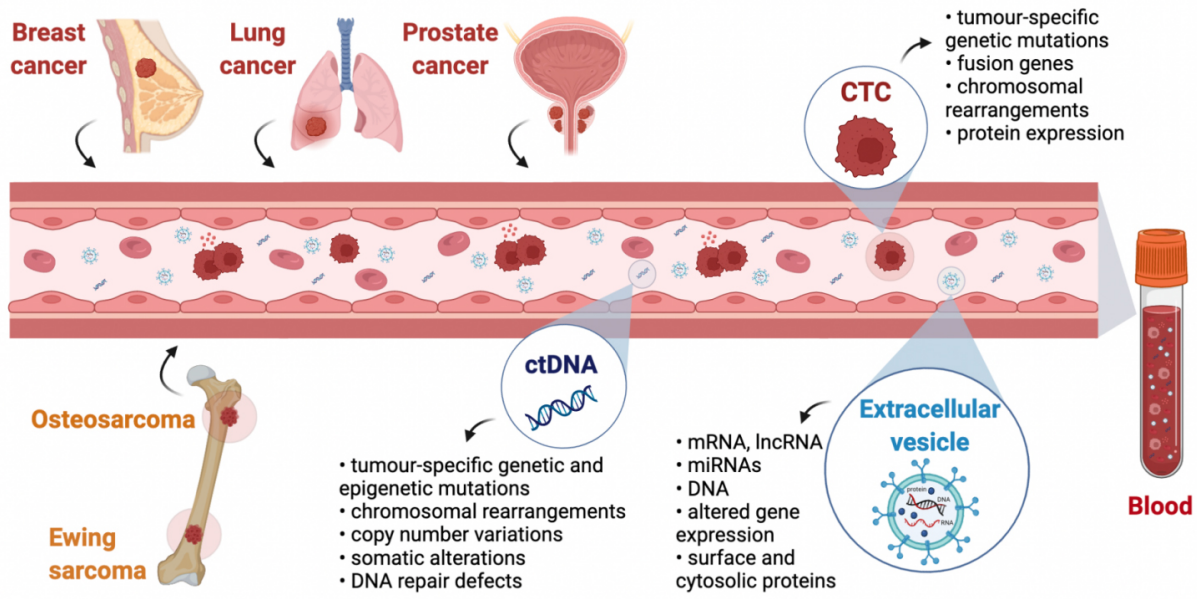

Figure 1. Liquid biopsies in primary and secondary bone cancers. Cell-free ctDNA, CTCs, and EVs are released into the general circulation by both primary (osteosarcoma and Ewing sarcoma) and secondary (breast, lung, and prostate) cancers and can be collected with a simple blood draw. Studying these three cancer-derived components by molecular or cellular analysis is termed liquid biopsy. Much information about the tumor can be achieved by analyzing CTCs, ctDNA, and EVs, making liquid biopsies a minimally invasive alternative to classical biopsies. Created with BioRender.com. ctDNA: Circulating tumor DNA; CTCs: circulating tumor cells; EVs: extracellular vesicles.

prostate cancer patients with visceral metastases had higher levels of ctDNA than bone metastatic patients[79].

CTCs for bone metastasis detection

As for CTCs, they have also been proven useful for diagnosis, prognosis, and monitoring treatment efficacy in metastatic breast cancer. Indeed, CTC quantification and characterization in patients with metastatic breast cancer have been associated with the presence of bone and liver metastases[80].

Remarkably, higher CTC counts correlated with multiple metastatic sites, while a lower CTC count was found in bone-only metastatic breast cancer patients, who also presented with a better prognosis[81,82], indicating that patients with less advanced disease had fewer CTCs. Moreover, patients with only one or two bone metastases had sharply fewer CTCs compared to patients with more bone metastases[82]. CTCs seem to have a subpopulation of metastasis-initiating cells that express epithelial cellular adhesion molecule, CD44, CD47, and c-MET. Once these cells were sorted and transplanted from a patient to immunocompromised mice, they induced bone, lung, and liver metastases[83].

CTC detection and quantification have shown prognostic potential in lung cancer patients, especially in advanced NSCLC, the most common histological subtype, highly metastasizing to bone. In the last decade, some studies showed that a high number of CTCs is a predictive and prognostic indicator of bone metastasis[84,85] in advanced lung cancer patients. The prognostic utility of CTCs in monitoring prostate cancer bone metastases was validated by multiple prospective studies performed on peripheral blood of metastatic castration-resistant prostate cancer patients after treatment. It was found that CTC counts ≥ 5 per 7.5 mL of blood are associated with lower overall survival and are predictive of bone metastases[86-88].

Some studies suggested a liquid biopsy approach involving simultaneous detection and quantification of both CTCs and ctDNA. A valid example to report is the COMET (NCT01745757) prospective study, conducted on peripheral blood samples collected before and after chemotherapy from a homogeneous group of HER2-negative breast cancer patients. In patients with bone, liver, and brain metastasis, CTCs were greater in number and ctDNA analyses revealed at least one mutation in tumor protein 53, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, and estrogen receptor 1 (*ESR1*) genes compared to non-metastatic patients[89]. These tumor-specific mutations in ctDNA analysis from plasma of patients with visceral and non-visceral metastasis, including bone, were confirmed in other studies[90-93], making this CTC-ctDNA signature potentially useful for diagnosis and prognosis of metastatic breast cancer.

Extracellular vesicles cargo and miRNAs for bone metastasis detection

As for EVs, starting from a meta-analysis conducted on publicly available microarray datasets derived from multiple-cancer type patients with and without bone metastasis, Bhadresha and colleagues identified 15 genes that were consistently upregulated in bone metastatic patients, and then validated their expression in patient serum-derived EVs. Among these, five were confirmed upregulated by qPCR in EVs isolated from the serum of breast and lung cancer patients harboring bone metastasis, namely, heat shock protein 90 alpha family class A member 1 (HSP90AA1), secreted phosphoprotein 1 (also referred to as osteopontin), IL-3, VEGFA, and protein tyrosine kinase 2. The authors concluded that this EV-derived mRNA gene signature could be a useful predictive tool for the early detection of bone metastases in breast and lung cancers[94]. Few studies focused their attention on EV-derived miRNAs released from lung cancer cells. A retrospective study was conducted on plasma-derived EV miRNAs from NSCLC patients, focusing on their potential as biomarkers of early detection and monitoring of bone metastasis. In particular, hsa-miR-574-5p, hsa-miR-328-3p, and hsa-miR-423-3p have been suggested as potential biomarkers for bone metastasis[95]. As for prostate cancer, EV-derived miR-181a-5p- was recently found upregulated in bone-metastatic prostate patients by Wang *et al.* and proposed as a biomarker for its diagnosis; similar findings were shown by Bryant *et al.* for miR-141 and miR-375[96,97]. Additionally, prostate microparticles, a type of prostate-specific EVs, were found to be more numerous in metastatic prostate cancer compared to non-metastatic cancer and showed better predictive ability than CTCs detected with the FDA-approved CellSearch system[98]. It is worth mentioning that a platform based on urine exosomal gene expression, termed ExoDX, was recently developed and tested in a utility trial on more than 500 patients. ExoDX-based scoring could predict high-grade prostate cancer in patients with uncertain prostate-specific antigen scores better than the gold standard (ROC AUC 0.7 *vs.* 0.62) and, conversely, it managed to predict which patients only had benign prostate hyperplasia, thus avoiding unnecessary biopsies[99].

Liquid biopsy in primary bone malignancies

ctDNA detection

Few studies have been performed on plasma-derived ctDNAs analysis from osteosarcoma patients[100,101]. One study focused on somatic mutations associated with tumor burden and disease outcome. Here, the researchers, using the targeted next-generation sequencing (NGS) approach, identified tumor-specific somatic alterations by comparing tumor and germline DNA extracted from peripheral mononuclear blood cells with tissue biopsies and demonstrated that patient-specific somatic alterations can be detected in ctDNA collected from the plasma samples at various stages of treatment, which allows the monitoring of disease burden[100]. In another study, Shulman and colleagues showed that ctDNA levels detected by NGS hybrid capture assay in peripheral blood samples of patients with newly diagnosed localized osteosarcoma and Ewing sarcoma may be associated with tumor burden, relapse, and negative disease outcomes. Interestingly, ctDNA analysis led to the identification of novel genomic features of osteosarcoma, including chromosome arm 8q copy number gains[101]. Specific and well-characterized genetic mutations, such as

stromal antigen 2 (STAG2) and TP53 loss-of-function mutations, translocation events, and fusion genes [Most commonly, EWSR1-FLI1 and EWSR1-erythroblast transformation specific-related gene (ERG)], have been found expressed in Ewing sarcoma patients, leading to the opportunity to monitor this bone malignancy through ctDNA[102]. The above-described retrospective study conducted by Shulman and colleagues showed an association between ctDNA detection in plasma samples and a poor clinical outcome in patients with newly diagnosed Ewing sarcoma and identified from ctDNA analysis additional genomic information, such as EWSR1 fusion and STAG2 loss-of-function mutations[101]. Another promising application for liquid biopsy in Ewing sarcoma was provided by Hayashi *et al.*, who found that tumor burden and response to therapy were related to increased levels of circulating *EWSR1-FLI1* fusion gene in plasma of patients. Moreover, they observed that *EWS-FLI1* levels in the circulation decreased after chemotherapy or surgery and then started to rise again during tumor recurrence[103].

CTCs analysis

CTCs have been proposed as potential predictive and prognostic markers for osteosarcoma metastasis[104]. A prospective study undertaken by Li *et al.* revealed a higher number of CTCs detected at baseline in peripheral blood of metastatic osteosarcoma patients compared to ones with localized disease. Moreover, they observed that CTC count was inversely correlated with the patient response after neoadjuvant chemotherapy[105]. Consistently, other preclinical studies have shown that CTC count variations after therapy or surgical resection can reflect the tumor's sensitivity to the treatment and may be a good indicator of metastasis[106,107], highlighting the clinical interest in dynamic monitoring of CTC changes for understanding treatment efficacy and detecting disease recurrence or metastasis in time. In particular, the presence of an increased percentage of CTCs with mesenchymal phenotype (identified by the epithelial-to-mesenchymal transition markers) in peripheral blood of a small group of osteosarcoma patients after chemotherapy treatment was associated with reduced disease-free survival, leading to the possibility to predict disease relapse and lung metastasis occurrence[106,107].

A few studies have reported the use of tumor-specific makers for CTC isolation and characterization in Ewing sarcoma patients, including CD99 expression and presence of chromosomal translocations, such as amplification of EWSR1-FLI1 transcript fusion gene, by using different methods[108,109]. Others have suggested that detection of CTCs at diagnosis in Ewing sarcoma patients may be associated with worse clinical outcomes and increased risk of recurrent disease or development of overt metastasis[109,110].

Extracellular vesicles cargo and miRNAs

EVs have recently been studied as diagnostic or prognostic serum biomarkers via a liquid biopsy approach in osteosarcoma. Circulating EVs RNA profiling of metastatic *vs.* primary osteosarcoma samples allowed the detection of multiple transcriptomic alterations in the former, providing a new clinically relevant approach to track metastatic osteosarcoma[111].

Several miRNAs, which are known to at least partially circulate inside EVs, with oncogenic or antitumor-suppressor roles in osteosarcoma, have been detected in the peripheral blood of patients. Some of them are emerging as important diagnostic and prognostic biomarkers, such as miR-148a[112], miR-574-3p, miR-214 and miR-335-5p[113], miR-49[1][114], miR-221[115], miR-191[116], and miR-421[117]. Conversely, miR-124[118], miR-101[119], and miR-195[120] were shown to be downregulated in the serum of osteosarcoma patients, compared to healthy individuals. A potential application for these findings could be to establish a predictive strategy for osteosarcoma prognosis using a combination of these miRNAs. Circulating miRNAs have also recently become the subject of study in Ewing sarcoma. As an example, a widely studied circulating miRNA related to Ewing sarcoma progression is miR-125b, which was found decreased in patients serum after surgical

resection when compared to healthy controls[121]. In the same study, its downregulation in the group of patients analyzed was also correlated with poor response to chemotherapy[121]. Recently, the research focus is shifting towards Ewing sarcoma-derived EV cargo as a prognostic biomarker source, particularly to their protein content. Samuel *et al.* identified and used CD99, histidine triad nucleotide-binding protein 1 (HINT1), and nerve growth factor receptor (NGFR) membrane proteins as potential biomarkers of Ewing sarcoma-derived small EVs. They developed an approach of immuno-enrichment of Ewing sarcoma-associated small EVs based on these EV surface proteins, for the subsequent detection of EWS-FLI1 and EWS-ERG fusion transcripts in EVs isolated from plasma of both localized and metastatic patients[122].

CLINICAL IMPLICATION OF LIQUID BIOPSY IN MONITORING DRUG RESISTANCE

Liquid biopsies in chemoresistance of primary and secondary bone cancers: an overview

A growing body of evidence suggests that the tumor secretome, including circulating-free DNA fragments from drug-resistant cells containing tumor-specific genetic and epigenetic mutations, is highly abundant in plasma; thus, the role of blood-based liquid biopsy is fundamental for this field of research[123]. Although several studies have been conducted on plasma samples of relatively small cohorts of patients, trying to identify resistance mutations that occur during treatment, the data obtained thus far are clinically informative about therapy response, but still not completely validated in clinical practice. According to the different types of tumors, quantification and analysis of ctDNA were found to be usable as a viable tool for this purpose[124].

A good example suggesting that ctDNA is a valuable strategy to monitor treatment efficacy was provided by Schiavon *et al.*, who showed that ESR1 mutations found in ctDNA from plasma of metastatic breast cancer patients previously treated with aromatase inhibitors are associated with resistance to endocrine therapy and shorter progression-free survival[125]. Additionally, liquid biopsy has also proved to be useful for the identification of biomarkers associated with cyclin-dependent kinase inhibitors (CDKi) resistance and for predicting the subsequent development of metastatic disease, in hormone receptor-positive/human epidermal growth factor receptor 2 negative (HR+/HER2-) advanced breast cancer patients. Patients treated with CDKi in combination with endocrine therapy presented with specific therapy-induced mutations in the ctDNA analyzed, including retinoblastoma, *ESR1*, fibroblast growth factor receptor 1, or phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha alterations[126-128], which could be useful for predicting disease outcomes and drive therapeutic decisions. It is also important to mention the detection and quantification of CTC acquired resistance, which may also act as a prognostic marker able to predict treatment outcomes[129]. Similarly, in castration-resistant prostate cancer patients treated with docetaxel, CTC count in blood was found to be an effective indicator of treatment sensitivity and patient survival[130].

Due to its high genetic instability, osteosarcoma treatment is often hampered by the acquisition of chemoresistance following therapy-induced selective pressure. Some studies observed that low serum levels of miR-375 in osteosarcoma patients were related to poor tumor response to preoperative chemotherapy[131]. Recently, other tumor-associated miRNAs have been linked with osteosarcoma chemoresistance, such as miR-491, which was found to be decreased in serum from osteosarcoma patients compared with healthy control subjects, and this decreased serum miR-491 level is correlated with increased metastasis, poor chemoresponse, and lower survival rate[114]. In contrast, the serum levels of miR-21 were found to be significantly higher in patients with osteosarcoma than in control subjects and correlated with advanced Enneking stage and chemotherapeutic resistance[132].

As for the development of chemoresistance in Ewing sarcoma, a recent study showed that circulating miR-125b levels were decreased in patient serum, and this was associated with poor response to chemotherapy[121].

Implication of extracellular vesicles in chemoresistance

Extracellular vesicles are emerging as key players in the transfer of drug resistance[133]; hence, they could be useful for monitoring the onset of this phenomenon during treatment.

Among the most studied constituents of tumor-derived EVs cargo, miRNAs have been identified as potential biomarkers for monitoring chemoresistance. EVs from doxorubicin-resistant osteosarcoma MG63 cells express high levels of the membrane transporter pump P-glycoprotein, transferring resistance to doxorubicin treatment to sensitive cancer cells horizontally[134]. In another report, miR-25-3p upregulation in the blood of osteosarcoma patients correlated with increased tumor growth and drug resistance[135].

Wei and colleagues demonstrated the role of EV-derived miR-222-3p detected in the serum of NSCLC patients for predicting gemcitabine sensitivity and identifying patients with the aggressive advanced and resistant disease[136]. In addition, higher levels of EV-derived miR-425-3p were found in the serum of NSCLC platinum-resistant patients compared with platinum-sensitive patients[137].

In breast cancer, it was found that EV-shuttled miR-222 released by doxorubicin-resistant breast cancer cells was locally transferred to M2 macrophages, thus activating their polarization. In these cells, overexpression of miR-222 suppressed phosphatase and tensin homolog gene, resulting in phosphorylation of Akt and activation of Akt signaling, which in turn supports cancer cells proliferation, migration, and invasion in a positive feedback loop. Accordingly, increased levels of miR-222 were found in EVs from plasma of patients presenting with chemoresistant breast cancer[138]. In another study, it has been demonstrated that the human osteotropic breast cancer cell line MDA-MB-231 treated with paclitaxel was able to release EVs enriched in the cell survival protein Survivin[139].

Interestingly, EVs can also act directly against anti-neoplastic agents. In fact, a recent study showed that HER2-positive breast cancer-derived EVs interfere with the activity of trastuzumab, acting as decoy receptors for it[140]. In fact, EVs secreted by those cancer cells were found to have HER2 on their surface, and trastuzumab administered systemically binds it, hence making the amount of antibody available to bind cells lower. Furthermore, higher levels of glutathione S-transferase P1 (GSTP1) mRNA were found by Yang *et al.* in EVs from the serum of non-responding breast cancer patients treated with neoadjuvant chemotherapy compared to the responders. Intriguingly, they also demonstrated that GSTP1-containing EVs transferred drug resistance horizontally, and hence proposed their use as negative predictive factors of chemoresistance and clinical outcomes in breast cancer patients treated with anthracycline/taxane-based therapy[141]. Similar results were observed for EV-bound transient receptor potential channel 5 mRNA found in peripheral blood of metastatic breast cancer patients, which could be a potential predictive marker of chemoresistance[142].

Kharaziha *et al.* conducted proteomic analysis on EVs derived from prostate cancer cells sensitive *vs.* resistant to docetaxel, identifying multidrug resistance protein 1 (MDR-1), MDR-3, endophilin-A2, and poly(A) binding protein 4 as proteins enriched in the latter as well as present in EVs from the serum of castration-resistant prostate cancer patients, suggesting that EVs may be used as biomarker candidates for predicting therapeutic response or resistance[143].

Larger longitudinal studies will be crucial to validate the biomarkers identified thus far, but the field holds great promise.

Factors hindering the clinical application of liquid biopsies

Although liquid biopsies are extremely promising tools, there are issues that need to be addressed before widespread clinical adoption can occur. First, since the techniques used are extremely sensitive, even small differences at the sample collection or processing level can cause significant differences in the final outcome. The use of serum instead of plasma, for example, can increase the amount of cell-free DNA released from other sources such as leukocytes, thus reducing the diagnostic ability of NGS-based assays, especially when trying to detect rare variants[144]. Moreover, lifestyle-related factors can affect the release of cell-free DNA in the general circulation, thus constituting an array of possible confounding factors that are hard to identify and characterize systematically[145]. As for CTCs, they are usually extremely rare and hard to capture, and although the CellSearch method has provided the field with a standardized method, CTCs captured this way are not viable. This means they can only be used for DNA and FACS/Immunofluorescence studies, but not for RNA-based assays or functional assays, including patient-derived xenografts or *in vitro* drug sensitivity tests[146,147]. Moreover, CTC analysis has some of the drawbacks as classical biopsies, since they are not necessarily representative of the entire tumor, but only a subpopulation of cells that were able to migrate and survive in the circulation. A possible solution under investigation to at least partially solve this issue is choosing different sites for the blood collection. It has been reported that arterial blood and blood withdrawn from a closer site to the primary tumor may provide a higher number of CTCs[148,149]. Significant efforts have been made by societies in both the United States and Europe to draw standard guidelines for preanalytical sample treatment, but while the consensus is widely accepted, liquid biopsies remain technically challenging for both the clinician and the analytical lab staff, which need specific training and facilities that are not always available locally, as well as training to interpret the results correctly[150]. In addition to the general problems outlined above, EVs carry their own challenges at the preanalytical level. They are the most recently recognized source of biological information, and therefore their development as liquid biopsy tool is still at an early stage. A key example is the issue of EV isolation. There are several methods that are currently available to isolate EVs, namely differential ultracentrifugation, isopycnic ultracentrifugation, size-exclusion chromatography, polymer-based precipitation, immune-capture, asymmetric field flow fractionation, ultrafiltration, and the countless possible combinations among them[151]. Unfortunately, no technique is absolutely superior to another, and, depending on the one investigator's use for EV isolation, the results obtained may vary[151]. Moreover, as stated above, EV secretion is stimulated by several factors that may relate to one's lifestyle, which may make the detection of tumor-specific exosomes challenging[70]. Finally, while these considerations are valid for oncology in general, the field of bone oncology is currently lacking specific clinical trials, although we are confident that it is just a matter of time before this gap is filled.

DISCUSSION AND CONCLUSIONS

Liquid biopsies have the potential to become one of the most powerful instruments in the clinical oncologist's toolkit, making diagnosis and prognosis increasingly accurate and therapy more personalized. In fact, the technique is rapidly gaining popularity, albeit still with somewhat limited success, as happens with most new implementations at early stages[39]. This is particularly important in bone oncology, where minimally invasive techniques to evaluate clinical response to therapy and prognosis are limited. Despite this, the field is currently lagging behind, and there is a significant gap that needs to be filled before concretely applying liquid biopsies in clinical practice. The potential benefits of liquid biopsies not only are related to the life quality and expectancy of cancer patients but also extend to the cost-effectiveness of treatments. In this regard, a cost-consequence analysis was recently conducted comparing the use of tissue biopsy alone *vs.* tissue biopsy-liquid biopsy combined diagnostic strategy, in NSCLC[152], where the

application of the latter implied lower overall medical expenses for the healthcare system compared to tissue biopsy alone. Additionally, liquid biopsies not only are a potential means of diagnosis but also can be used as risk-profiling tools, which could be able to identify subjects that are at risk for bone metastases, so that preventive therapies can be initiated. Of course, CTCs have been the most widely used in preclinical and clinical practice thus far, gaining FDA approval for use in some metastatic tumor prognosis (i.e., colorectal, breast, and prostate cancer), mainly due to the development of well-standardized isolation and analytical techniques. Nevertheless, ctDNA and tumor-derived EVs may become even more important than CTCs in the future, especially for personalized medicine. Indeed, they are easier to detect and characterize and can genetically reflect the tumor as a whole, providing the potential for real-time monitoring of tumor progression and development of chemoresistance, as has also been proposed or demonstrated in other neoplasias[153-155]. Liquid biopsies could also be useful in tracking metastases before they become overt and therapy resistance by implementing them in standard follow-up protocols and analyzing the emergence of mutations that are important for therapy resistance and metastasis, as the pioneering work by the Bardelli group already demonstrated in colorectal carcinoma, although timelines may need to be readapted[153,154]. Moreover, EVs also play an active role in malignancy and chemoresistance, and targeting them could be a valuable tool in the oncologist's toolkit, especially considering that some of the most commonly used EV secretion inhibitors are clinically approved for other conditions (manumycin A, D-pantethine, imipramine, tipifarnib, neticonazole, climbazole, ketoconazole, and triademenol)[156] and could be repurposed to reduce EV-induced chemoresistance. We believe this research area is largely underexplored and will see an increase of interest in the next few years, also considering the advances in next-generation sequencing that could eventually lead to a "single-EV sequencing", which would really open a new avenue for the field[157].

DECLARATIONS

Authors' contributions

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Reviewed the manuscript: Ucci A, Rucci N, Ponzetti M

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Aberrant calcium signalling downstream of mutations in TP53 and the PI3K/AKT pathway genes promotes disease progression and therapy resistance in triple negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is characterized as an aggressive form of breast cancer (BC) associated with poor patient outcomes. For the majority of patients, there is a lack of approved targeted therapies. Therefore, chemotherapy remains a key treatment option for these patients, but significant issues around acquired resistance limit its efficacy. Thus, TNBC has an unmet need for new targeted personalized medicine approaches. Calcium (Ca^{2+}) is a ubiquitous second messenger that is known to control a range of key cellular processes by mediating signalling transduction and gene transcription. Changes in Ca^{2+} through altered calcium channel expression or activity are known to promote tumorigenesis and treatment resistance in a range of cancers including BC. Emerging evidence shows that this is mediated by Ca^{2+} modulation, supporting the function of tumour suppressor genes (TSGs) and oncogenes. This review provides insight into the underlying alterations in calcium signalling and how it plays a key role in promoting disease progression and therapy resistance in TNBC which harbours mutations in tumour protein p53 (TP53) and the PI3K/AKT pathway.

Keywords: Triple-negative breast cancer, TP53, PI3K/AKT pathway, calcium

INTRODUCTION

Triple-negative breast cancer is associated with a worse disease outcome

Breast cancer (BC) is the most common female-specific cancer in the world[1]. Triple-negative BC (TNBC) accounts for ~15% of all BC cases and is characterized by a clinically more aggressive disease, which is linked with higher recurrence rates, increased metastatic potential and poorer overall survival[2-4]. Unlike oestrogen receptor-positive (ER+) BC and human epidermal growth factor receptor 2 (HER2)-positive BC, TNBC lacks the expression of oestrogen, progesterone and HER2 receptors[5,6]. In both ER+ and HER2-positive BC, the expression at the protein and/or gene level of these receptors has been successfully targeted as a cancer treatment with either small molecule inhibitors or monoclonal antibodies[7]. The results of these interventions in ER+ and HER2-positive BC have resulted in both improved response rates and overall survival of those BC patients[8,9]. In TNBC, the development of targeted approaches to treat the disease lags behind other BC subtypes meaning that TNBC has one of the poorest survival rates of all breast cancer subtypes[10,11].

In TNBC, chemotherapy remains one of the main treatment options for patients, but acquired resistance to chemotherapy remains a significant clinical problem[12]. In recent years, advances in treating TNBC patients with novel targeted agents have shown benefits. For example, for patients who have a germline breast cancer gene (BRCA) 1/2 mutation, poly(ADP-Ribose) polymerase (PARP) inhibitors are used to treat TNBC patients in both the adjuvant and metastatic settings[13-15]. However, patients with *BRCA* mutations account for only a small percentage of TNBC cases. Immunotherapy offers new hope for TNBC patients: results from the recent IMPASSION study demonstrate that immunotherapy combined with chemotherapy offers benefit to a subset of patients who have elevated programmed death ligand (*PDL*) 1 expression[16]. Lastly, tumour-associated calcium signal transducer 2 (TROP2), encoded by the (*TACSTD2*) gene, is a transmembrane glycoprotein expressed in approximately 80% of TNBC. The antibody-drug conjugate (ADC) sacituzumab govitecan, a TROP2-directed antibody and topoisomerase inhibitor drug conjugate, has been approved for the treatment of TNBC by the FDA[17]. However, there remains a significant proportion of TNBC patients for whom these therapies offer little benefit.

Calcium (Ca^{2+}) is an essential component required for normal cellular function and is involved in the regulation of processes such as metabolism, muscle contraction and phagocytosis as well as cell growth, proliferation and apoptosis[18,19]. Local Ca^{2+} concentrations oscillate with varying frequency and amplitude, enabling the induction or modulation of signal transduction and gene transcription[20-22]. In line with this, it has become evident that Ca^{2+} supports the functions of key tumour suppressor genes (TSGs) and oncogenes commonly altered in BC, such as tumour protein p53 (TP53), phosphatase and tensin homolog (PTEN) and Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), as well as a number of others reviewed elsewhere[23]. Generally, oncogenes promote cellular survival by dampening Ca^{2+} signalling, whereas TSGs induce apoptosis through Ca^{2+} influx[23]. Owing to this key role, it is unsurprising that Ca^{2+} is altered downstream of key driver genes, enabling cancer progression and treatment resistance[18,24,25]. Consequently, Ca^{2+} represents an area of interest for the development of new drug therapies targeted to specific genomic alternations[26-28]. This review outlines in detail the mechanisms of Ca^{2+} modulation and how these mechanisms are altered in specific TNBC cohorts who harbour mutations in TSGs such as TP53 and PTEN and oncogenes such as PIK3CA.

The role of calcium in cancer

Ca^{2+} plays a key role in cell growth, proliferation and apoptosis through rapid local fluctuations in intracellular calcium (Ca_i^{2+}), as well as long-term genomic changes by regulating signal transduction and gene transcription. In non-excitable cells such as epithelial cells in BC, Ca_i^{2+} is predominately modulated through store-operated calcium (SOC). Here, store-operated calcium channels (SOCCs) allow the influx of Ca^{2+} into the cytosol due to differential Ca^{2+} concentrations which are established and tightly regulated through the continual action of various pumps and calcium channels[18,24,29]. Extracellular Ca^{2+} is maintained at a concentration of 2 mM and cytosolic Ca^{2+} at a concentration of 100 nM[30]. In addition, the ER and mitochondria act as Ca^{2+} stores, holding Ca^{2+} concentrations of ~1 mM and ~200 nM, respectively[31]. These differential calcium gradients allow for localised changes in Ca^{2+} through the action of calcium channels, which in turn promotes various functions such as signal transduction and gene transcription as well as cellular processes such as proliferation and apoptosis.

SOC is initiated by Ca^{2+} release from the endoplasmic reticulum (ER) through associated calcium channels such as inositol trisphosphate (IP3) receptors (IP3Rs) or ryanodine receptors (RyRs). IP3R activation is initiated by upstream activation of plasma membrane G protein-coupled receptors (GPCRs) and receptor tyrosine kinase (RTK)[32]. Following activation of these receptors by their associated ligands, phospholipase C (PLC) enzyme is produced, hydrolysing phosphatidylinositol 4,5-bisphosphate (PIP2) and resulting in the production of diacylglycerol (DAG) and IP3, the latter of which activates IP3R inducing ER Ca^{2+} release[33]. In addition, the channel can also be activated by common cellular stresses found in the tumour microenvironment (TME), such as reactive oxygen species (ROS), ER stress, altered cellular energetics, hypoxia and drug treatment[23]. Alternatively, RyR releases Ca^{2+} from the ER upon sensing changes in intracellular Ca^{2+}[34]. Subsequently, the decrease in store Ca^{2+} is detected by a family of stromal interaction molecule (STIM) channels[35], which mediate store-operated calcium entry (SOCE) primarily through calcium release-activated calcium modulator (ORAI) and occasionally transient receptor potential (TRP) channels at the plasma membrane[36-38]. Normal Ca^{2+} concentrations are subsequently restored through Ca^{2+} efflux via the plasma membrane calcium pump (PMCA) and Na^+/Ca^{2+} exchanger (NCX) along with reabsorption into the ER through the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). In addition, it has been increasingly found that the membrane potential of cancer cells has become more depolarised, and as such voltage-gated calcium channels (VGCC) have also been shown to contribute to calcium entry[39,40]. Furthermore, these channels have also been shown to contribute to SOCE in cancer cells[41].

Research has continued to demonstrate that aberrant Ca_i^{2+} is a common feature in cancer that is able to promote neoplastic transformation and drug resistance[20,42,43]. This is mediated through altered calcium channel expression or activity, enabling cancer hallmarks such as migration/invasion, proliferation, survival and apoptotic resistance[25,27,28,44]. Typically, increased Ca_i^{2+} enhances proliferation, while a reduction and faster recovery are linked to apoptotic resistance and decreased sensitivity to chemotherapeutic agents[45]. To date, several studies have shown various calcium channel families are altered in BC and linked to tumorigenesis[31]. Importantly, this work has also uncovered specific differences in channel expression between BC subtypes[46]. For example, IP3R2 and IP3R3 have been found to be upregulated in TNBC tissue compared to luminal subtypes. SOCE is also altered in TNBC through increased ORAI1 and STIM1 expression, which promotes invasion and migration, while in patient samples, it was linked to poorer prognosis[47,48]. In contrast, ORAI3 was found upregulated in luminal and HER2 BC[49]. SOCE inhibitors in early preclinical studies have demonstrated an ability to inhibit proliferation and migration in MDA-MB-231 TNBC cell lines and reduce tumour growth in TNBC mouse models[50]. In addition, Azimi *et al.* (2017) observed that TRPC1 is significantly increased in TNBC compared to all other BC subtypes, resulting in an enhanced epithelial-mesenchymal transition (EMT) phenotype[51]. Going forward, this review focuses on the role of Ca^{2+} in supporting TSGs and oncogenes commonly altered in TNBC; however, altered calcium

channel expression in BC subtypes has been extensively reviewed elsewhere[24,46,52].

The role that calcium plays on the impact of TP53 mutations in TNBC

TNBC is a heterogeneous disease, which includes distinct molecular subtypes[6,53-55]. This heterogeneity partly explains the limited impact that targeted therapeutic approaches have made in treating the majority of TNBC patients. Part of this heterogeneity is associated with the mutational background of TNBC, which could impact a patient's response to therapeutic intervention. However, this could provide opportunities for scientists and clinicians to design novel approaches to treat TNBC.

Somatic mutations in the TSG TP53 (located at 17p13.1) occur in ~80% of TNBC cases[56,57]. Normally, TP53 is activated due to various stress signals such as DNA damage, hypoxia, ROS, oncogenic activation and cancer treatment[23]. In this way, TP53 helps prevent tumorigenesis by regulating biological processes such as apoptosis, cell cycle, DNA repair and senescence[58]. Mutations that result in loss of TP53 activity and impair its normal cellular functions have been linked to chemoresistance as well as reduced overall survival[59-64]. Most (90%) functionally relevant TP53 mutations produce missense products that have both loss- and gain-of-function features[65,66] and are more resilient to degradation[57]. Most known cancer-associated TP53 coding DNA sequence (CDS) changes are within the DNA-binding domain corresponding to exons 5-8 and amino acids 102-292, with > 28% of these nonsynonymous SNPs occurring at eight key sites[67]. Many of these originate from aberrant CpG methylation[67]. Notably, recurrent TP53 aberrations during cancer growth result in selective sweeps of new clones with independent mutant TP53 proteins[68].

In relation to Ca^{2+}, TP53 is known to have a non-transcriptional role in the cytosol, inducing apoptosis in cells by regulating Ca^{2+} release from the ER[69,70]. Recent research has shown that wild-type TP53 localises to ER and mitochondria-associated membranes (MAMs), where it interacts with SERCA increasing Ca^{2+} loading in the ER by enhancing its activity[71,72] [Figure 1A]. In addition, TP53 also promotes ER Ca^{2+} transfer to the mitochondria inducing pro-apoptotic mitochondrial overload, leading to the release of pro-apoptotic factors[72].

Mutant TP53 disrupts these processes, leading to apoptotic resistance and reduced sensitivity to chemotherapy[69,72,73]. Here, TP53 fails to induce SERCA activity, reducing mitochondrial Ca^{2+} response, which also reduces caspase 3 and PARP cleavage, thus promoting an anti-apoptotic phenotype[72] [Figure 1B]. In addition, Giorgi *et al.* (2015) demonstrated that wild-type TP53 cells were sensitive to doxorubicin (Adriamycin) treatment, as TP53 mediated an increase in cleavage of PARP and caspase 3, which resulted in reduced cell survival[72]. However, in TNBC TP53 mutant MDA-MB 468 cell lines, doxorubicin failed to increase both SERCA activity and Ca^{2+} levels, thus conferring chemotherapy resistance[72]. This critical role of TP53 in promoting pro-apoptotic Ca^{2+} in treatment sensitivity was further confirmed by Giorgi *et al.*, who demonstrated that overexpression of SERCA or mitochondrial calcium uniporter (MCU) restored treatment sensitivity[74]. This effect was lost when Ca^{2+} levels were reduced by chelation, highlighting how Ca^{2+} is integral to the function of TP53[74]. In addition, mouse xenograft tumours established using mouse embryo fibroblast (MEF) H-ras induced cells were observed to double in size with TP53 loss compared to wild-type controls, corresponding with an observed decrease in Ca_i^{2+} Activity[74].

Other calcium channels such as TRP have also been linked to TP53 pro-apoptotic Ca_i^{2+} changes in BC. TRP channels promote calcium entry at the plasma membrane following activation by PLC, DAG or store release from IP3R channels[75]. These channels support store refilling via SOCE through the interaction with ORAI and STIM[76]. A novel anti-neoplastic organic derivative GaQ3 (which has undergone phase 1 clinical trials in patients with solid tumours) was demonstrated to induce apoptosis by promoting TP53 expression

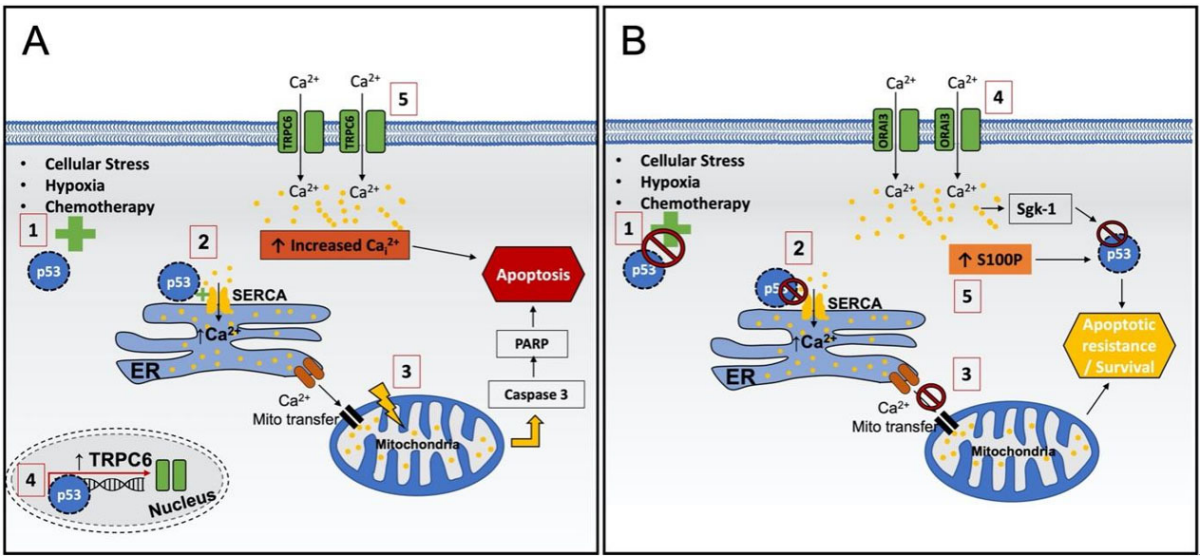

Figure 1. Summary of wild-type and mutant p53 calcium mediated cell death processes. (A) Wild-type p53: (1) induces apoptosis by different cellular stresses such as hypoxia and chemotherapy; (2) mediates an increase in ER Ca^{2+} through SERCA; (3) promotes ER Ca^{2+} transfer to the mitochondria resulting in a Ca^{2+} overloading activated caspase 3 and thus PARP; and (4) mediates apoptosis through an induction in TRPC6 expression and (5) associated Ca^{2+} influx. (B) (1) Mutations in TP53 result in a loss of function; (2) TP53 fails to induce SERCA; (3) this leads to reduced mitochondrial Ca^{2+} promoting apoptotic resistance; (4) ORAI3 is increased in TNBC, promoting Ca^{2+}-mediated increase in serum and glucocorticoid-induced protein kinase-1 (SGK-1) inducing TP53 degradation and (5) increased S100 calcium-binding protein P (S100P) also induces TP53 degradation. Both enhance cell survival. ER: Endoplasmic reticulum; PARP: poly(ADP-Ribose) polymerase; SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase.

through elevated Ca_i^{2+} concentrations[77,78]. The key role of Ca^{2+} in this mechanism was confirmed using the Ca^{2+} quencher TMB-8, which prevented an increase in Ca_i^{2+}, leading to an inhibition of TP53-mediated apoptosis. Further work found that this increase in Ca_i^{2+} was mediated by the binding of TP53 to the *TRPC6* promoter, resulting in the overexpression of TRPC6, enhancing Ca^{2+}-dependent apoptosis in MCF-7 BC cells[78]. A putative TP53 binding site on the *TRPC6* promoter was also identified by bioinformatic analysis using the Mat Inspector module of the genomatix database, suggesting TP53 is a potential regulator of TRPC6[78]. Thus, it was identified that the Ca_i^{2+} released through TRPC6 is mediated by TP53 and plays a major role in promoting apoptosis in a range of cancer cell line models including MCF-7[77,78].

Research in a range of BC subtypes including TNBC has also observed that altered SOC channel expression is linked to apoptotic and chemotherapy resistance through the TP53 pathways[23,79]. A recent study demonstrated through bioinformatic analysis that ORAI3 was elevated in patients with poor response or residual disease following chemotherapy treatment, which was also predictive of poor patient outcomes[80]. Increases in ORAI3 expression induced resistance to the chemotherapeutic drugs cisplatin, 5-FU and paclitaxel by reducing apoptosis and increasing survival of T47D and MCF7 BC cell lines[80]. These effects were found to be mediated by ORAI3, promoting a decrease in TP53 and cyclin-dependent kinase inhibitor 1A (p21). This was confirmed by the removal of extracellular Ca^{2+} and/or Orai3 functionality, which resulted in an increase in TP53 expression and reduced chemoresistance. Interestingly, Hasna *et al.* (2018) also discovered that the Phosphoinositide 3-Kinase (PI3K)-Protein Kinase B (AKT) pathway was also induced by the observed increase of ORAI3 expression in chemoresistant cells[80]. PI3K inhibitors partially inhibited resistance induced by TP53 expression. This chemoresistant effect was mediated by PI3K induction of the SGK-1, leading to TP53 degradation via MDM2 proto-oncogene (Mdm2) and Nedd4-like E3 ubiquitin-protein ligase (Nedd4-2). Prior to this work, Brickley *et al.* (2013) highlighted a link between SOCE and SGK-1 in TNBC cell lines[81]. SOCE activation induced the expression of SGK-1 following

exposure to cellular stress. This mechanism appeared cytoprotective, as siRNA targeting SGK-1 under the same conditions increased apoptosis.

Proteins such as S100P are also linked to TP53, where their expression was found to be altered in BC, enabling drug resistance[82]. Specifically, the expression of S100P is elevated in TNBC and linked to chemotherapy resistance and poor survival[83-86]. Gibadulinova *et al.* identified reduced phosphorylation and activity of TP53 in response to DNA damage following S100P binding in a range of cancer cell lines including BC cell lines MCF7 and T47D[87]. When bound to TP53 protein, S100P promotes cell survival and resistance towards anticancer drugs such as paclitaxel and cisplatin, evading senescence and thus promoting cancer progression[87].

Until recently, it has been challenging to develop drugs that can target TP53 for cancer treatment[88]. This is primarily because TP53 expression is lost in ~10% of cases, and in the other cases, the function of the mutant protein is changed. However, advances in drug design have enabled both scientists and clinicians to develop drugs such as Eprenetapopt (APR-246) and COTI-2, which aim to reactivate TP53. These TP53 reactivators have been tested in preclinical studies in TNBC[89] but also in clinical trials of TP53-mutant myelodysplastic syndromes (MDS)[90] and solid tumours (NCT02433626). Further investigation is warranted to ascertain if these drugs impact any of the calcium signalling mechanisms mentioned previously.

Taken together, there is compelling evidence that Ca^{2+}-dependent TP53 apoptosis and associated mechanisms contribute to poor outcomes in TNBC and resistance to chemotherapy. Consequently, it highlights calcium channels as a therapeutic target to modulate Ca^{2+} concentrations in mutant TP53 TNBC, which, through TP53 restoration or calcium channel activation drugs, could lead to improved responses to existing treatments and better overall patient survival.

Calcium signalling in PI3k/AKT and PTEN pathways in TNBC

PI3K/AKT oncogenic signalling through pathway activation is linked to cell survival, growth and apoptosis[91]. Alterations in this pathway are common in TNBC and are associated with poorer outcomes and treatment resistance[92]. Up to 30% of TNBC tumours harbour aberrations in the oncogenic PI3K/AKT pathway through mutation of either AKT serine/threonine kinase 1 (AKT1) or PIK3CA[93,94]. The loss of wild-type TP53 regulation of AKT (located at 14q32.33), PIK3CA (at 3q26.32) and PTEN (at 10q23.31) has consequences for the PI3K-AKT pathway. PIK3CA encodes an alpha subunit of Phosphatidylinositol 3-Kinase (PI3K). PTEN is another TSG whose mutation can mimic the effects of mutant TP53[95]. PTEN is a negative regulator of the PI3K/AKT pathway through downregulation of phosphorylated AKT, and it is also commonly altered in TNBC[94,96].

TP53, AKT, PIK3CA and PTEN changes together constitute the genetic basis of the majority of TNBC cases and initiate the initial stages of tumour development[97]. Mutations at these four genes may be oncogenic on their own, or may occur in tandem with other TSG mutations: PIK3CA, PIK3R1 (encoding regulatory protein p85α) and PTEN commonly co-occur in BC[98]. Changes at AKT, PIK3CA and PTEN are much less common than those at TP53[61]. PIK3CA mutations are typically missense and constitute ~10%-18% of cases, and in ~4% of cases, PIK3R1 may be mutated[99,100] - often, PIK3CA and PIK3R1 co-occur independently[101]. PTEN is mutated in ~6%-7% of cases and AKT1 is rarer at ~3%[100]. Although all 4 genes may have cancer-related SNPs, TP53 tends not to have other mutation types, in contrast to AKT and PIK3CA that can be amplified or possess CNVs; additionally, PTEN tends to be inactivated, often by deletion.

There is an established link between the PI3K/AKT pathway and TP53 [Figure 2]. RTKs are usually activated and phosphorylated by growth factors and hormones. PI3K, consisting of p110 (α,β,γ,δ) and p85, is recruited to the RTK leading to phosphorylation of PtdIns (4,5) P2 to PtdIns (3,4,5) and subsequent recruitment of PDK1 to AKT at the PH domain. PDK1 is reliant on activation by PI3K. Activation of the AKT signalling pathway results in regulation of the cell cycle, promoting cell growth, survival and migration and inhibiting apoptosis[102]. $mTORC_2$ activates AKT, thus leading to cell proliferation and survival. $mTORC_1$ is activated by phosphorylated AKT and promotes protein translation and cell growth[103,104]. TP53 has three primary domains - transactivation domain, DNA-binding domain and tetramerization domain[105]. TP53 is regulated by the MDM2 proto-oncogene (Hdm2) protein via binding and physical separation from target genes, resulting in low levels of TP53 in normal cells. TP53 regulates PI3K via binding and inhibition of PIK3CA, which encodes p110α, and reduces AKT activation. Mutations in TP53 may lead to hyperactivation of PIK3CA and the PI3K signalling pathway[99]. Studies have demonstrated the regulatory effects of TP53 on PTEN via the functional TP53 binding site present within the PTEN promoter region, resulting in inhibition of PtdIns(4,5)phosphorylation and subsequent AKT activation. AKT can enhance the function of Hdm2 via phosphorylation, and evidence suggests that PTEN may prevent the degradation of TP53 through suppression of AKT activation[106]. PTEN has also been identified as a stabiliser of TP53 by way of physical association between the two, conferring protection from degradation to TP53. Despite these stabilising functions, deletion of PTEN and subsequent activation of the PI3K pathway have been shown to activate TP53, with significantly increased TP53 expression present in PTEN-/- cells[106].

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TP53 can regulate cell survival via inhibition of the PIK3CA as well as the PI3K/AKT pathway, independent of PTEN. This transcriptional downregulation of PIK3CA via TP53 has been demonstrated in head and neck cancer cell lines, where the PTEN protein was not detected and induction of TP53 resulted in decreased PIK3CA expression and reduced AKT phosphorylation, indicating the transcriptional regulatory effects of TP53 on PIK3CA[107]. A study investigating the mechanisms involved in the regulation of PIK3CA transcription via TP53 in ovarian cancer described two promoters present on the *PIK3CA* gene that have been shown to directly bind TP53 resulting in transcriptional inhibition. It was found that suppression of TP53 resulted in increased levels of p110α transcripts, proteins and PI3K signalling pathway activity, while overexpression of TP53 resulted in decreased levels of p110α protein[108]. Loss-of-function mutations in TP53 correlate with hyperactivation of PIK3CA and the PI3K pathway, resulting in worse prognoses for head and neck squamous cell carcinoma patients[99].

Research has demonstrated that the PI3K/AKT pathway can modulate Ca^{2+} release from ER stores via interaction with IP3R[23] [Figure 3A]. This calcium channel is found on the ER membrane, where it controls the frequency and amplitude of Ca^{2+} oscillations to either promote cell survival or induce apoptosis[109]. Under hypoxia conditions or chemotherapy treatment, IP3R promotes Ca^{2+} transfer to the mitochondria, inducing the release of pro-apoptotic factors[23]. However, the anti-apoptotic protein AKT has been shown to phosphorylate IP3R, preventing ER Ca^{2+} transfer to the mitochondria and inhibiting apoptosis[110,111] [Figure 3B]. This, in turn, enables resistance to chemotherapy and promotes survival during hypoxia[110,111]. The PI3K/AKT pathway is also negatively regulated by the TSG PTEN[112], which is commonly lost or mutated in TNBC and associated with poorer prognosis as well as treatment resistance[113,114]. PTEN is known to localise at the ER and reduce AKT-induced phosphorylation of IP3R, thus promoting ER Ca^{2+}

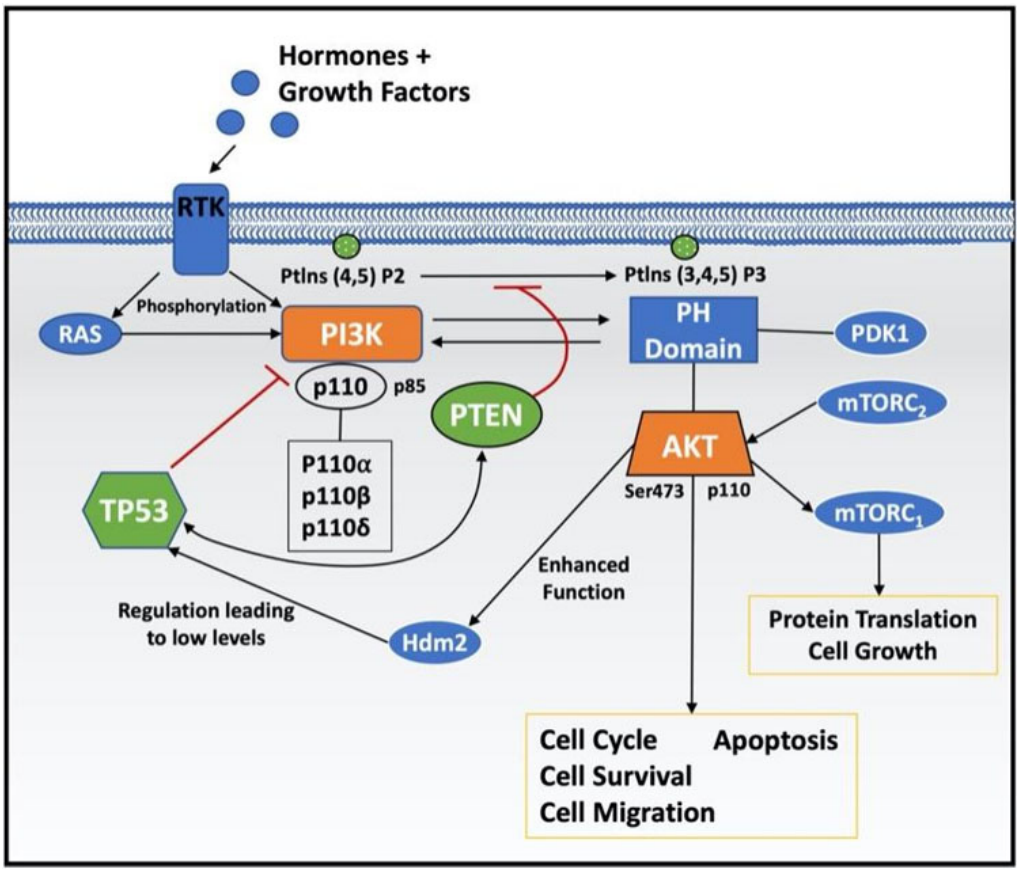

Figure 2. The role of TP53 in the PI3K/AKT signalling pathway. RTKs are activated by hormones and growth factors, leading to the recruitment and phosphorylation of PI3K. PDK1 is then recruited to AKT at the PH domain. Cell cycle, survival, migration and apoptosis regulation occur as a result of AKT signalling pathway activation. AKT is also activated by $mTORC_2$ and $mTORC_1$ is activated by phosphorylated AKT, promoting protein translation and cell growth. Hdm2 regulates P53 in normal healthy cells, resulting in low levels. P53 regulates PTEN, inhibiting PI3K phosphorylation and AKT activation. AKT may enhance the function of Hdm2 and PTEN may prevent P53 degradation via AKT pathway inhibition. P53 binds to PI3K to inhibit PIK3CA and mutations in TP53 may result in PIK3CA hyperactivation and subsequent PI3K signalling pathway activation. AKT: Protein kinase B; PTEN: phosphatase and tensin homolog.

release. In addition, it also associates with MAM and regulates the transfer of Ca^{2+} to the mitochondria, enabling Ca^{2+}-mediated apoptosis[115]. In PTEN mutant BC, IP3R becomes phosphorylated and inactivated by AKT, decreasing Ca^{2+} release from the ER and associated transients to the mitochondria, reducing the sensitivity to Ca^{2+}-mediated apoptosis[115]. This work highlights a potential link between PTEN loss and AKT activation, supporting treatment resistance and disease progression via deactivated IP3R mediated apoptotic resistance.

In relation to IP3R, its ability to mediate Ca^{2+} release from ER can be finetuned through interaction with calcium binding proteins such as neuronal calcium sensor-1 (NCS-1)[116]. Recent studies in TNBC cells have shown that overexpression of NCS-1 increases Ca^{2+}, promoting survival[117]. Furthermore, this mechanism was linked to paclitaxel resistance, where NCS-1 binding to IP3R promotes drug resistance[116,118] [Figure 3B]. Interestingly, researchers found IP3R2 and IP3R3 expression was elevated in breast tumours compared to adjacent normal cells[119]. Furthermore, Singh *et al.* (2017), analysing MCF-7 cells, demonstrated that IP3R inhibition is able to induce autophagy and subsequent cell death, an effect not observed in non-tumorigenic cells[119]. In addition, IP3Rs are also associated with the nuclear envelope and nucleoplasmic reticulum, where I3PRs can also regulate Ca^{2+} levels within the nucleus to promote cell

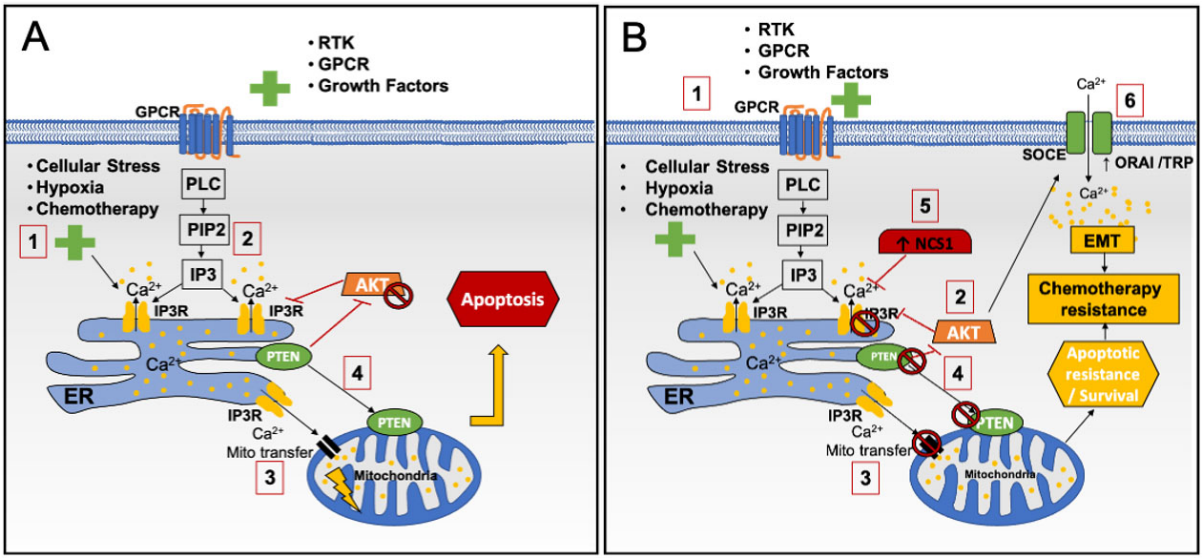

Figure 3. Role of calcium store release via IP3R in mediating apoptosis and its regulation via PI3K/AKT pathways. (A) (1) G protein-coupled receptors (GPCR), Receptor tyrosine kinase (RTK) and others, along with cellular stress, can induce PLC, PIP2 and IP3, leading to activation of IP3R channels on the ER, promoting Ca^{2+} store release. (3) This mediates Ca^{2+} transfer to the mitochondria inducing apoptosis. (4) PTEN also plays a role in mediating this pathway in part by blocking anti-apoptotic oncogene AKT. (B) (1) Activation of oncogenes such as AKT inhibits IP3R Ca^{2+} release from ER stores under chemotherapy and GPCR activation. (2) This is achieved by phosphorylating IP3R and (3) reducing Ca^{2+} transfer to the mitochondria, enabling apoptotic resistance. (4) PTEN loss is common in TNBC, enabling AKT signalling. (5) NCS-1 is increased in TNBC, promoting IP3R inactivation as well. (6) SOCE is mediated after store release via IP3R; channels such as ORAI and TRP that are activated have increased expression in TNBC and are linked to promoting EMT and thus chemotherapy resistance. RTK: Receptor tyrosine kinase; Orai: calcium release-activated calcium channel protein; PLC: phospholipase C; GPCR: G protein-coupled receptors.

proliferation[120,121]. In TNBC, reducing nuclear IP3R Ca^{2+} by chelation decreased cell proliferation and induced tumour necrosis[122]. Overall, this research identified that IP3R can impact both PI3K/AKT dependant and independent pathways.

Ca^{2+} store release via IP3R, which is modulated by PI3K-AKT/PTEN pathways, has an indirect contribution to drug-resistant mechanisms by association with Ca^{2+} binding proteins. For example, Ca^{2+}/calmodulin-dependent kinase (CAMKK) is essential for AMP-activated protein kinase (AMPK) induction following activation by epidermal growth factor (EGF) in TNBC cell lines[123]. AMPK is an essential regulator of AKT activation under cellular stress such as drug treatment and hypoxia[123]. Ca^{2+} is a requirement for this mechanism as its chelation using BAPTA-2AM completely inhibits AMPK-induced phosphorylated AKT. Specifically, AMPK phosphorylates s-phase kinase-associated protein 2 (Skp2) at S256, triggering the formation of an E3 ligase complex that promotes activation of AKT[123]. This outlined mechanism was shown to promote cell survival and apoptotic resistance. In BC patient samples, Skp2 activation correlates with increased AKT and AMPK expression and is associated with poor survival outcomes. In addition, Han *et al.* (2018) found that targeting AMPK-Skp2 in TNBC MDA-MB-231 cell lines increased their sensitivity to anti-EGF receptor-targeted therapy[123]. Furthermore, this study also showed in the same TNBC cell lines that this mechanism is not only induced by EGF but also hypoxia, promoting survival under such conditions. Hypoxia is a common characteristic in the tumour microenvironment (TME), which is also linked to chemotherapeutic resistance[73].

SOCE mediated through ORAI and STIM as well as TRP enables the refiling of ER stores following PI3K-IP3R induced release, which supports signal transduction. Both ORAI and STIM have been shown to be

upregulated in TNBC, enabling disease progression and promoting treatment resistance[124]. Using TNBC MDA-MB-231 cell lines, Bhattacharya *et al.* further demonstrated that (in the presence of phosphorylated AKT) ORAI3- and STIM1-mediated Ca^{2+} promotes the induction of Snail family transcriptional repressor (SNAIL)[125]. Induction of SNAIL expression is known to mediate EMT, a cellular phenotype linked to drug resistance[126,127]. In addition, TRPC1 has also been shown to play a role in EMT of TNBC via regulation of hypoxia-inducible factor 1 alpha (HIF-1α) and AKT signalling in PTEN-deficient BC cells[51]. Azimi *et al.* also noted that TRPC1 has a prognostic value in basal BC a molecular subtype associated with TNBC[51]. Specifically, high TRPC1 expression was associated with significantly poorer patient relapse-free survival[51]. Furthermore, it was noted that TRPC1 can induce EMT and promote chemotherapy resistance in MDA-MB-468 TNBC cell lines through the upregulated ATP-binding cassette, subfamily C, member 3 (ABCC3), a multidrug resistance ATP-binding cassette (ABC) transporter[128]. In addition, this channel, alongside ORAI1 and IP3R2, has been found to be upregulated following doxorubicin treatment in MDA-MB-231 TNBC cells[129].

TRPC5 is another family member whose overexpression is linked to chemotherapy resistance by induction of a protective autophagy mechanism which promotes cancer cell survival[130]. Adriamycin (ADM/doxorubicin) was found to induce an increase in Ca_i^{2+} which was reduced with siRNA targeting of TRPC5. Exploration of the underlying pathway discovered that Ca^{2+} mediated through TRPC5 could activate CaMKKβ, AMPKα and mammalian target of rapamycin (mTOR), which resulted in the induction of autophagy[130]. As noted above, AMPK modulates AKT activation, which can regulate ER store release, and this can then facilitate chemotherapy resistance. In the study by Zhang *et al.* (2017), targeting the TRPC5-mediated CaMKKβ/AMPKα/mTOR pathway increased sensitivity to ADM[130]. Furthermore, analysis of patient samples via a tissue microarray (TMA) found that TRPC5 was significantly increased in patient samples post chemotherapy treatment when compared to pre-treated samples. Supporting the role of TRPC5 in chemotherapy resistance, studies have linked TRPC5 expression to the induction of expression of the multidrug resistance protein 1 (MDR1) in TNBC cells[18].

Because of the large number of TNBCs that have an activated PI3K/AKT pathway, it is unsurprising that early phase clinical trials of both PI3K inhibitors such as buparlisib[131,132] and AKT inhibitors such as capivasertib[133] and ipatasertib[134] have been conducted in this setting. Interestingly, these trials have reported clinical activity and benefits to patients. The identification of key biomarkers of response to these treatments and their study in combination with chemotherapy or other targeted therapies warrants further investigation.

Furthermore, as discussed above, there is a strong link between Ca^{2+} signalling and those TNBC cancers that have a TP53 mutation and/or activation of the PI3K/AKT pathway. Activation of the PI3K/AKT pathway can be achieved through either mutation of PIK3CA/AKT1 or loss of expression of PTEN. Our review highlights that several calcium channels associated with Ca^{2+} release from ER and subsequent SOCE are linked to the activation of the PI3K/AKT pathway. Aberrations in the PI3K/AKT pathway disturb Ca^{2+} handling and enable escape mechanisms such as cell survival and apoptotic resistance, thus promoting chemotherapy resistance.

Current approaches to target calcium channel signalling in cancer

The role of calcium channels in mediating therapy resistance through interplay with genomic alternations in TNBC highlights calcium channels as potential targets for personalized medicine approaches. The study of calcium channel blockers (CCBs) is an area of intense research[26-28,135]. Several calcium blocking drugs have been developed which can modulate calcium channels and be exploited as cancer treatments[27,43,135,136].

Calcium channels provide a plethora of targets to modulate Ca_i^{2+}, and ion channel targeting drugs are currently the second largest group of FDA-approved drugs, therefore providing a bank of new drugs which can be repurposed for the treatment of cancer[137].

Several studies have identified existing FDA-approved drugs that can modulate store-operated release and entry through key channels such as ORAI, STIM, TRP and IP3R, as outlined herein, thus representing potential future therapeutics for the tackling disease progression and chemotherapy resistance in the TNBC cohorts outlined. For example, an extensive screen of 1118 unique FDA-approved drugs discovered 11, mainly cardiac glycosides, which have the ability to modify Ca^{2+} signalling, promote Ca^{2+}-calmodulin kinase (CamK) activity and reverse the suppression of TSGs such as secreted frizzled related protein 1 (SFRP1), tissue inhibitor of metallopeptidase-3 (TIMP-3) and WNT inhibitory factor 1 (WIF-1), leading to cell death of colorectal cells[26]. Other similar studies identified the ability of the immunosuppressant drugs to regulate SOCE, namely, leflunomide used to treat arthritis and teriflunomide used for multiple sclerosis[138]. In addition, the drug eflornithine, also known as DFMO, has been found to reduce SOCE and promote a decrease in cell proliferation and cell death resistance in colorectal cancer[139]. Eflornithine has been found to inhibit TRPC1 expression, which, as outlined above, plays a role in AKT signalling in PTEN deficient TNBC.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed anti-inflammatory, anti-pyretic drugs that have demonstrated anti-cancer activity[140]. The metabolite of aspirin, salicylate, has been found to inhibit SOCE and mediate mitochondrial uncoupling, reducing mitochondrial Ca^{2+} uptake and leading to a decrease in the proliferation of colon cancer cells[141]. Furthermore, other NSAIDs such as ibuprofen and indomethacin have also been shown to modulate SOCE through STIM1, which decreased colorectal cancer growth[142]. The CCB mibefradil, previously used to treat hypertension, can also inhibit SOCE by blocking ORAI channels, promoting cell apoptosis and cell cycle arrest[143]. In addition, a potent synthetic oestrogen used in the treatment of prostate and breast cancer, diethylstilbesterol, can inhibit SOCE via TRPM7 channels[144,145]. The potential to target SOCE modulation for disease benefit has led to the development of a number of drugs, such as CM2489, which has entered into phase 1 clinical trial for psoriasis; if successful, it could prove useful for TNBC treatment[146]. CM2489 is a first-in-class calcium release-activated calcium (CRAC) channel inhibitor that targets ORAI channels. Lastly, IP3R channels appear to be a key target in PI3K/AKT pathways mediating chemoresistance; both caffeine and heparin have been shown to inhibit these channels and could hold future potential as novel treatments in combination with existing anti-cancer agents[147,148].

CONCLUSION

Targeting calcium channel signalling and key oncogenic pathways is a novel therapeutic approach to treating TNBC

TNBC is characterized as an aggressive form of BC associated with poor patient outcomes. For the majority of patients, there is a lack of approved targeted therapies. TNBC has frequent genomic alternations in TP53 and the PI3K/AKT pathway. This review provides insight into the underlying alterations in Ca_i^{2+} mediated through calcium channels and how this plays an important role in promoting disease progression and therapy resistance in TNBC harbouring mutations in key TSG and oncogenes. Specifically, TNBC with mutant TP53 is associated with a loss of Ca^{2+} store release via SECRA, leading to reduced mitochondrial Ca^{2+} loading; this, in turn, promotes cell survival and apoptotic resistance, thus enabling treatment resistance. In addition, I3PR-mediated ER store release and SOCE modulation are altered by PI3K/AKT activation, promoting apoptotic resistance. Consequently, the outlined evidence highlights calcium channels as therapeutic targets to modulate altered Ca_i^{2+} downstream of common genomic alterations in TNBC.

This promise is supported by the fact that a growing number of calcium targeting drugs are under development, as well as a number of existing FDA-approved drugs. However, owing to the complexity of calcium signalling, its ubiquitous nature and differential expression between cell types and genomic alterations, care needs to be exercised when targeting Ca^{2+} signalling. It may be that targeting Ca^{2+} signalling, whilst beneficial for one cancer, could be detrimental for another. Overall, this review highlights Ca^{2+} electroporation as an emerging area in TNBC that could hold significant potential as disease biomarkers as well as future therapeutics when combined with treatments that can inhibit the PI3K-AKT pathway or reactivate TP53 expression.

DECLARATIONS

Authors' contributions

Conception and design: Buchanan PJ, Eustace AJ, Downing T
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Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Exosome secretion from hypoxic cancer cells reshapes the tumor microenvironment and mediates drug resistance

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Abstract

Hypoxia is a common phenomenon in solid tumors as the poorly organized tumor vasculature cannot fulfill the increasing oxygen demand of rapidly expanding tumors. Under hypoxia, tumor cells reshape their microenvironment to sustain survival, promote metastasis, and develop resistance to therapy. Exosomes are extracellular vesicles secreted by most eukaryotic cells, including tumor cells. They are enriched with a selective collection of nucleic acids and proteins from the originating cells to mediate cell-to-cell communication. Accumulating evidence suggests that exosomes derived from tumor cells play critical roles in modulating the tumor microenvironment (TME). Hypoxia is known to stimulate the secretion of exosomes from tumor cells, thereby promoting intercellular communication of hypoxic tumors with the surrounding stromal tissues. Exosome-mediated signaling pathways under hypoxic conditions have been reported to cause angiogenesis, invasion, metastasis, drug resistance, and immune escape. Recently, the programmed cell death ligand-1 (PD-L1) has been reported to reside as a transmembrane protein in tumor exosomes. Exosomal PD-L1 was shown to suppress T cell effector function in the TME and cause drug resistance to immune checkpoint therapy. This review provides an update about the pivotal role of tumor-derived exosomes in drug resistance to chemotherapy and immunotherapy, particularly under hypoxic conditions. Emerging strategies that target the exosomes in the hypoxic TME to enhance the antitumor efficacy are discussed.

Keywords: Hypoxia, drug resistance, exosome, tumor microenvironment, immunotherapy, non-coding RNA

INTRODUCTION

Hypoxia is a well-known hallmark of solid tumors when the tumor vasculature cannot provide adequate oxygen to support the aggressive growth of rapidly expanding tumors. Preclinical studies demonstrated that hypoxia mediates resistance to various modalities of cancer therapy, including chemotherapy, radiotherapy, and immunotherapy[1,2]. Tumor hypoxia may also promote invasion and metastasis. Extensive evidence is also available from clinical investigations to suggest that highly hypoxic tumors are associated with treatment failure, increased incidence of distant metastases, and dismal disease-free and overall survival[3].

Exosomes are a unique form of extracellular vesicles with endosomal origin and sizes ranging from 30 to 100 nm. They are secreted from diverse cell types upon the fusion of multivesicular bodies with the plasma membranes[4]. Exosomes mediate intercellular crosstalk by transferring mRNAs, microRNAs (miRNAs), and proteins from donor to recipient cells[5,6]. Cargoes loaded in exosomes are biologically active when taken up by the recipient cell, and they lead to various downstream functions[7]. Tumor-derived exosomes have been shown to facilitate the intercellular transfer of pro-tumorigenic factors in the tumor microenvironment (TME)[8,9]. They promote angiogenesis, invasion, and proliferation in recipient cells to support tumor growth and a pro-metastatic phenotype. In a recent proteome profiling study of exosomes derived from human primary and metastatic colorectal cancer cells, selective enrichment of metastatic factors and signaling pathway components was observed[10]. In glioma, exosomes have been reported to convey signals between the tumor and TME to facilitate bidirectional communication[11]. Hypoxia is known to stimulate the secretion of exosomes from tumor cells, thereby promoting cell-to-cell communication between the hypoxic tumors and the surrounding stromal tissues. Exosomal cargoes are also altered under hypoxic conditions to stimulate angiogenesis, invasion, metastasis, therapeutic resistance, and immune escape[12].

In this article, we summarize the critical role played by hypoxic tumor-derived exosomes in tumor progression and resistance to cancer therapy. Novel approaches to target the exosomes in the hypoxic TME to potentiate the anticancer efficacy are discussed.

HYPOXIC TME

TME is a dynamic and complex system around a tumor, which is composed of blood vessels, fibroblasts, extracellular matrix fibers, immune cells, and signaling molecules, supporting proliferation, metastasis, and therapy resistance of tumor cells[13]. The rapid proliferation of cancer cells and aberrant blood vessel formation create hypoxic conditions in malignant tumors. Hypoxia is considered a hallmark of TME, which is generally defined by a low oxygen tension of below 10 mmHg[14]. It is also known to orchestrate the various malignant phenotypes of cancer cells by activating multiple oncogenic signaling pathways. At the molecular level, the hypoxic TME is largely regulated by the hypoxia-inducible factor (HIF) family of transcriptional factors[15]. Accumulating evidence suggests that tumor-derived exosomes play a critical role in invigorating the interaction between cancerous and non-cancerous cells in the hypoxic TME to propel cancer progression[16].

EFFECT OF HYPOXIA ON TUMOR-DERIVED EXOSOME

Induction of exosome release

TME-associated cells have been shown to secrete more exosomes than normal cells to promote intercellular communication and nutrient exchange[17]. In the clinical setting, the number of circulating exosomes

collected from the blood of cancer patients was estimated to be more than two-fold higher than that of healthy subjects[18]. Granulocytic myeloid-derived suppressor cells (G-MDSCs) in tumors were reported to produce more exosomes than those found in the spleen[19]. Indeed, more secretion of exosomes from cancer cells has been reported in breast cancer[20], colorectal cancer[21], gastric cancer[22], glioma[23], hepatocellular carcinoma[24], and pancreatic cancer[25]. Interestingly and in contrast, hyperoxia (excessive oxygen tension) has been reported to reduce the number of exosomes released from colorectal cancer cells[19]. It is noteworthy that the induction of exosome secretion in hypoxia may be universal because more exosomes were also released from non-cancerous cells (including bone, cardiac, and kidney cells) under hypoxic conditions[26-28]. To date, the detailed mechanism leading to increased exosome release in hypoxia is still not clear. Nevertheless, two Rab GTPases (Rab27a and Rab27b) were shown to promote exosome secretion by facilitating the docking of the multivesicular bodies at the plasma membrane[29], whereas Rab7 was reported to direct exosomes to lysosomes for degradation[30]. In ovarian cancer cells, hypoxia was shown to upregulate Rab27a but downregulate Rab7 to promote exosome release[31].

Increase in exosomal heterogeneity

Exosomes secreted from different cells contain different cargoes and markers. On the other hand, exosomes released from the same cell line can carry different constituents[18]. While this heterogeneity is advantageous for exosome applications, it poses obstacles to a thorough understanding of exosome biology[32,33]. Depending on the cell state, exosomes of different sizes and carrying different cargoes are secreted[34]. To this end, exosomes secreted from glioma cells were shown to reflect the hypoxic status, and they mediate hypoxia-dependent activation of neovascularization during tumor development[34]. The heterogeneous exosome population could have a different functional effect on the recipient cells[35,36].

Production of smaller exosome

The size of exosomes released from mammalian cells is known to vary considerably in individual cells because of their unique exosome biogenesis process[36]. Interestingly, it has been reported that exosome size is associated with different disease states. In non-small cell lung cancer (NSCLC) patients, a smaller exosome detected in the pulmonary vein is associated with a shorter time to relapse and shorter overall survival after curative surgery[37]. It is generally believed that hypoxia tends to release smaller exosomes. Relatively smaller exosomes have been reported in different cancer cell lines, including colon[21], pancreatic[25], and prostate[38] cancer cells. Under hypoxic conditions, it is believed that the supply of cellular materials for membrane synthesis may not meet the demand for exosome production, thus leading to the secretion of smaller exosomes[12]. It is also hypothesized that smaller exosomes could be transmitted more easily via the blood circulation to metastatic sites to form the pre-metastatic niches because hypoxia changes the hemodynamics in the tumor vasculature[12]. Moreover, smaller exosomes could be internalized faster and more efficiently than larger exosomes in the recipient cells[39]. An ongoing prospective clinical trial (NCT02310451) is underway, which examines exosome size and various other parameters as potential biomarkers in melanoma patients.

Influence on exosomal cargo sorting

It is known that exosome content is not simply reflecting the cellular content of its origin. Some sorting mechanisms are in place to facilitate specific cargo sorting processes. Hypoxia has been shown to affect the sorting of the following three major cargoes (i.e., nucleic acids, proteins, and lipids) into exosomes.

Nucleic acids

The effect of hypoxia on the nucleic acid content in tumor-derived exosomes has recently been summarized in a few excellent reviews[40,41]. It is noteworthy that only limited studies have reported the effect of hypoxia on cancer-derived exosomal DNA. On the other hand, the alteration and biological effects of non-coding

RNAs (ncRNAs) in tumor-secreted exosomes under hypoxia have been extensively studied. Representative examples are summarized in Table 1. Various mechanisms have been proposed to regulate the expression of ncRNAs in hypoxia. For the HIF-dependent mechanism, the binding of HIF-1α and/or HIF-2α to the hypoxic response element (HRE) of the gene promoter of miR-155 has been reported[42,43]. Moreover, miRNAs are differentially sorted into exosomes according to their specific sequence or motif[44]. A few RNA binding proteins, such as SYNCRIP, were shown to direct specific miRNAs sharing a common extra-seed sequence hEXO motif for enrichment in the exosomes[45]. Moreover, hypoxia also affects RNA alternative splicing[46,47] and RNA editing[48], which could make specific miRNAs more suitable for loading into tumor-derived exosomes. On the other hand, hypoxia was also reported to regulate a few facilitators which load nucleic acids into exosomes. The RNA binding proteins YBX1 and hnRNPA1 were reported to mediate the selective loading of miR-133 and miR-1246, respectively, into tumor-derived exosomes under hypoxia[49,50].

Hypoxia has been shown to regulate the expression of several ncRNAs, which affect the expression of HIF-1α and subsequently form positive or negative feedback loops to modulate the hypoxic TME[51,52]. In hypoxic gastric cancer cells, such a positive feedback loop involving miR-301a-3p, PHD3, and HIF-1α has been reported[53]. miR-301a-3p was upregulated in hypoxic gastric cancer cells and the tumor-secreted exosomes[53]. miR-301a-3p was subsequently shown to suppress the hydroxylase PHD3 and thus promote the protein stability of HIF-1α to maintain the hypoxic response. In pancreatic cancer, the hypoxic induction of a HIF-1α-stabilizing circular RNA (cirZNF91) in cancer-secreted exosomes was reported to promote chemoresistance in normoxic cancer cells[54]. Upon transmission to normoxic cells, circZNF91 was found to bind competitively to miR-23b-3p, subsequently abolishing the inhibition of miR-23b-3p on its target Sirtuin1 (SIRT1). The upregulated SIRT1 was shown to increase the deacetylation-dependent stability of HIF-1α protein, thus promoting glycolysis and chemoresistance in the recipient normoxic cancer cells[54].

Proteins

Similar to nucleic acids, proteins loaded into tumor-derived exosomes are not necessarily proportional to the cellular protein composition[55]. However, the precise mechanism contributing to specific exosomal protein sorting remains obscure. It has been postulated that hypoxia could influence the protein loading process of tumor-derived exosomes by regulating ubiquitination. Ubiquitinated proteins are recognized by ubiquitin-binding domains within multivesicular endosomes for degradation, thus limiting the amount of membrane available for exosome formation[56]. To this end, hypoxia is commonly known to control protein ubiquitination and ubiquitination-associated enzymes[57].

Cell membrane glycoproteins are also known to participate in cell-to-cell and cell-environment communication[58]. Interestingly, the expression profile of glycoproteins in tumor-derived exosomes is different from that of healthy cells[59]. Moreover, glycoprotein expression could be affected by hypoxia[60]. As glycans are known to regulate protein sorting and uptake into exosomes, hypoxic cells have been shown to take up more exosomes in a proteoglycan-dependent manner[61].

Proteins secreted in tumor exosomes are also known to participate in hypoxia-associated responses. In breast cancer, metastasis-associated protein 1-loaded exosomes were reported to transfer in between cancer cells to regulate the response to hypoxia and estrogen signaling[62]. Taken together, hypoxia promotes numerous malignant phenotypes of cancer cells by altering the exosome protein heterogeneity, whereas proteins in tumor-derived exosomes could contribute to the hypoxic response. Table 2 summarizes the representative exosomal protein cargoes and other constituents that are preferentially secreted from hypoxic tumors to module the TME.

Table 1. Representative non-coding RNA cargoes secreted in hypoxic cancer cell-derived exosomes to modulate the tumor microenvironment

Non-coding RNA	Donor cell	Recipient cell	Biological function (mechanism)	Reference
let7a	Melanoma	Macrophage	Induce M2 polarization of TAM and promote oxidative phosphorylation to support cancer growth (downregulation of insulin-AKT-mTOR signaling)	[121]
linc-RoR	Hepatocellular carcinoma	Hepatocellular carcinoma	Promote cancer cell proliferation; mediate chemoresistance (induction of PDK1 and HIF-1α protein expression by suppressing miR-145)	[78]
lncRNA UCA1	Bladder cancer	Bladder cancer	Promote cancer growth; stimulate migration and invasion (induction of EMT)	[95]
miR-10a	Glioma	MDSC	Activate MDSC (regulation of RORA/IκBα/NF-κB signaling)	[113]
miR-21	Glioma	MDSC	Activate MDSC (regulation of PTEN/PI3K/AKT signaling)	[113]
miR-23a	Lung cancer	Endothelial cells	Promote angiogenesis; increase vascular permeability (inhibition of the propyl hydroxylases PHD1 and PHD2, thereby stabilizing HIF-1α protein; inhibition of the tight junction protein ZO-1)	[92]
miR-24-3p	Nasopharyngeal carcinoma	T cell	Inhibit T cell proliferation and differentiation (inhibition of FGF11; upregulation of p-ERK, p-STAT1, and p-STAT3)	[103]
miR-25-3p	Breast cancer	Breast cancer and macrophage	Promote cell proliferation and migration (stimulation of IL-6 secretion from macrophage via NF-κB signaling)	[94]
miR-125b-5p	Ovarian cancer	Macrophage	Induce M2 polarization (regulation of SOCS4/5-STAT3 pathway)	[120]
miR-210	BMSC	Lung cancer	Promote metastasis (induction of STAT3 to mediate EMT)	[73]
miR-301a-3p	Pancreatic cancer	Macrophage	Induce M2 polarization (downregulation of PTEN and activation of PI3K/Akt signaling)	[118]
miR-5100	BMSC	Lung cancer	Promote metastasis (induction of STAT3 to mediate EMT)	[97]

BMSC: Bone marrow-derived stem cell; EMT: epithelial-mesenchymal transition; linc-RoR: long intergenic non-protein coding; RNA: regulation of reprogramming; lncRNA: long non-coding RNA; MDSC: myeloid-derived suppressor cells; TAM: tumor-associated macrophage.

Table 2. Exosomal protein cargoes and other constituents are preferentially secreted from hypoxic tumors to modulate the tumor microenvironment

Cancer type	Type of cargo	Biological function and mechanism	Reference
Colorectal cancer	Wnt4 protein	• Intercellular communication with normoxic cancer cells • Exosomal Wnt4 promoted the translocation of β-catenin to the nucleus in normoxic cells • Activation of β-catenin signaling enhanced cancer cell motility and invasion	[137]
Glioblastoma	TSP1, VEGF, LOX protein	• Promote cancer growth, angiogenesis, and metastasis	[138]
Glioblastoma	MMPs, IL-8, caveolin 1, PDGFs, and lysyl oxidase	• Induce angiogenesis *in vitro* and *ex vivo* through phenotypic modulation of endothelial cells • Stimulate endothelial cells to secrete potent growth factors and cytokines and to activate pericyte PI3K/Akt signaling to promote migration	[34]
Lung cancer	TGF-β1	• TGF-β1 downregulates NKG2D on the cell surface of NK cells to suppress NK cell cytotoxicity	[116]
Nasopharyngeal carcinoma	MMP13	• Promote migration and invasion	[139]
Nasopharyngeal carcinoma	HIF-1α	• Promote EMT to induce migration and invasion	[140]
Prostate cancer	Tetraspanins (CD63 and CD81), heat shock proteins (HSP90 and HSP70), and Annexin II	• Remodel the epithelial adherens junction pathway to enhance invasiveness and stemness of naïve prostate cancer cells	[38]
Prostate cancer	Lactic acid	• Under chronic hypoxia, prostate cancer cells secrete more exosomes as a survival mechanism to remove metabolic waste	[141]

IL-8; Interleukin-8; LOX: protein lysine 6-oxidase; MMP: matrix metalloproteinase; PDGF: platelet-derived growth factor; TSP1: thrombospondin-1; VEGF: vascular epithelial growth factor.

Lipids

Various lipid components are important building blocks for exosome membranes. They include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, cholesterol, ceramide, sphingomyelin, and glycosphingolipids[59]. They play critical roles in the biogenesis of exosomes. Sphingomyelin is hydrolyzed to ceramide, which promotes the budding of multivesicular bodies from the endosomes[63]. On the other hand, ceramide is metabolized to sphingosine 1-phosphate, which interacts with the inhibitory G protein-coupled receptors to induce exosome biogenesis[64]. Interestingly, knockdown of a key cholesterol lipid efflux transporter ABCG1 was found to inhibit cancer growth, concomitantly with the intracellular accumulation of exosomes and the exosomal cargo[65]. Phosphatidylserine has been reported to play a critical role in facilitating the uptake of exosomes secreted from hypoxia-induced stem cells by human umbilical cord endothelial cells[66]. Under hypoxic conditions, triglyceride was found to accumulate in prostate cancer cells and the secreted exosomes. Importantly, these exosomes were shown to promote cancer proliferation and invasion following reoxygenation[67]. Hypoxia is known to upregulate ceramide expression, which is proposed to promote the exosome release[68].

Effect on exosome uptake by recipient cells

Little is known about the effect of hypoxia on the intercellular transfer and uptake of tumor-derived exosomes from other components within the TME. A hypoxic environment is known to promote glycolysis and lactic acid production. Excess production of lactic acid results in acidic pH in the hypoxic TME. Given that an acidic environment is more suitable for the stability and isolation of exosomes[69], the intracellular transport of exosomes may benefit from a hypoxic and acidic TME. It has also been proposed that the uptake of cancer-secreted exosomes by the recipient cells was found to be more efficient under hypoxia[70]. The smaller exosomes secreted from hypoxic tumors, as described above, may facilitate the more efficient intercellular transfer. However, this hypothesis has not been verified by a detailed experimental investigation. On the other hand, hypoxic tumor cells are also known to take up more exosomes from the surroundings[61]. Upon the recognition and binding of exosomes to recipient cells, the former is internalized by various processes, including endocytosis via clathrin, caveolae, or lipid raft-dependent manner. Under hypoxic conditions and the closely associated acidic cellular environment, exosome uptake could be promoted by lipid raft-dependent endocytosis[61], caveolin-dependent endocytosis[71], and phagocytosis[72].

REGULATION OF TME BY HYPOXIC TUMOR-DERIVED EXOSOMES

As described in the previous section, hypoxia significantly alters the properties of exosomes secreted from cancer cells. Accumulating evidence indicates that the altered tumor exosomes are responsible for the reshaping of TME, thereby promoting cancer cell proliferation, chemoresistance, metastasis, and angiogenesis [Figure 1].

Promotion of cancer proliferation and chemoresistance by hypoxic tumor-derived exosomes

Within the hypoxic TME, cancer cells are known to secrete pro-tumorigenic molecules in the exosomes to promote cancer survival and proliferation. miR-210 is one of the most extensively studied hypoxia-induced miRNAs driving cancer progression[73]. In breast cancer, the abundance of miR-210 was reported to be remarkably higher in the exosomes derived from hypoxic cancer cells than those from normoxic ones, which allows the cells to sustain survival under hypoxia[74]. A set of differentially expressed exosomal miRNAs has been identified in the exosomes secreted from patient-derived melanoma cells under hypoxic culture conditions[75]. Hypoxia was found to upregulate miR-494-5p, miR-4497, miR-513a-5p, and miR-6087 but downregulate miR-125b-5p, miR-21-5p, and miR-3934-5p in the exosome[75]. Interestingly, the alteration of miRNAs was closely associated with cancer survival according to bioinformatics pathway analysis[75]. Exosomes secreted by human glioma were also shown to promote the differentiation of neural stem cells into astrocytes[76]. Transcripts related to cell proliferation and astrocyte differentiation were found

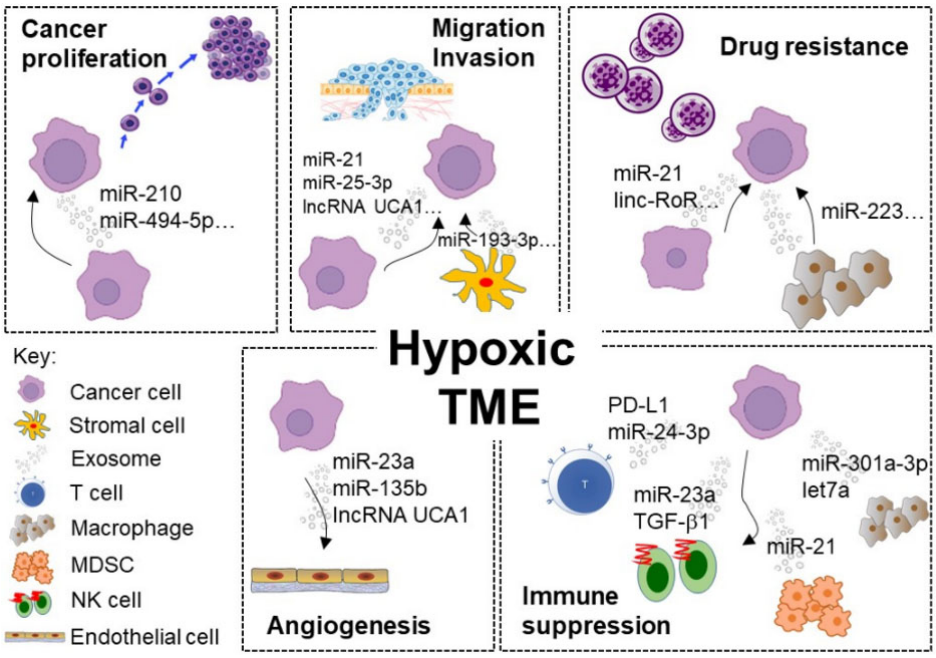

Figure 1. Representative examples of exosome-mediated intercellular communication within the hypoxic TME driving cancer progression, chemoresistance, and immune suppression. (1) Cancer proliferation: Hypoxic cancer-secreted exosomes were enriched with miRNAs supporting cell survival (e.g., miR-210) in the neighboring cancer cells. (2) Drug resistance: Numerous ncRNAs (e.g., linc-RoR and miR-21) were transferred via exosomes from hypoxic and resistant cancer cells to sensitive cells and induced drug resistance. (3) Migration and invasion: exosomes containing various ncRNAs (including lncRNA UCA1 and miR-193-3p) facilitate cancer–cancer or cancer–stromal intercellular communication to stimulate migration and invasion by modulating EMT. (4) Angiogenesis: ncRNAs (including miR-23a and lncRNA UCA1) were enriched in the exosomes secreted from hypoxic tumor cells to promote tumor vascular endothelial cell proliferation and angiogenesis in HIF-1α-dependent or -independent pathway. (5) Immune suppression: Exosomes enriched with miRNAs (e.g., miR-23a and let-7a) and other immunosuppressive molecules (e.g., PD-L1 and TGF-β1) were secreted from hypoxic tumors to promote an immunosuppressive TME. TME: Tumor microenvironment.

to be remarkably upregulated in human mesenchymal stem cells when co-cultured with glioma-secreted exosomes[76]. Hypoxic tumor-secreted exosomes may represent an important therapeutic target that mediates the aggressiveness of glioma.

Hypoxia is known to mediate chemoresistance by regulating the cell cycle, autophagy, cell senescence, and drug efflux transporters. In recent years, the emerging role of hypoxic cancer cell-derived exosomes in reshaping the TME and causing chemoresistance is also revealed. In NSCLC, hypoxic cancer-derived exosomes have been shown to induce cisplatin resistance in normoxic cancer cells through the transmission of miR-21[77]. The transfer of miR-21 from hypoxic cell-derived exosome to normoxic cancer cells was demonstrated to downregulate PTEN and the PI3K/Akt pathway, which subsequently induced cisplatin resistance[77]. In hepatocellular carcinoma, the abundance of a stress-responsive lncRNA (linc-RoR) was significantly increased in hypoxic cancer-derived exosomes than in their normoxic counterpart[78], which is associated with resistance to sorafenib and doxorubicin. Linc-RoR was shown to induce TGF-β, thereby suppressing chemotherapy-induced cell death but promoting tumor-initiating cell proliferation[78]. Stromal cells, such as cancer-associated fibroblasts (CAFs) in the TME, could also mediate chemoresistance in cancer cells. miR-223 was upregulated in TAMs and TAM-derived exosomes under hypoxia[79]. miR-223 loaded in hypoxic exosomes was shown to reduce apoptosis and induce drug resistance in ovarian cancer by downregulating PTEN and thus activating PI3K/Akt signaling[79].

Moreover, exosomes have also been shown to mediate the transfer of the drug-resistant phenotype. Drug-sensitive cancer cells have been shown to become drug-resistant following the incorporation of exosomes shed from drug-resistant cancer cells[80-84]. Moreover, exosomes were shown to be involved in the intercellular transfer of functional ABCB1 (P-gp) from multidrug-resistant donor cells to drug-sensitive recipient cells[81,85-87]. Furthermore, exosomes have also been reported to mediate drug resistance by exporting specific drugs via the exosome pathway[88] and neutralizing antibody-based chemotherapy[89].

Induction of cancer angiogenesis by hypoxic tumor-derived exosomes

The induction of angiogenesis by hypoxia has been extensively studied[90]. More recently, accumulating evidence demonstrates that hypoxic tumor-derived exosomes played a significant role in angiogenesis. In malignant glioblastoma multiforme, exosomes derived from cancer cells under hypoxia were shown to induce angiogenesis by stimulating cytokine and growth factor secretion from endothelial cells, subsequently promoting pericyte migration[34]. In pancreatic cancer, the exosomal lncRNA UCA1 secreted from cancer cells under hypoxic conditions was shown to promote angiogenesis via a miR-96-5p/AMOTL2/ERK1/2 pathway[91]. In lung cancer, miR-23a secreted in tumor-derived exosomes in hypoxia was reported to target the key HIF-1α regulators (propyl hydroxylases PHD1 and PHD2), thereby sustaining the overexpression of HIF-1α and promoting angiogenesis[92]. Moreover, hypoxia-induced exosomal miR-23a was also shown to inhibit the tight junction protein ZO-1 and increase vascular permeability[92]. It is noteworthy that most studies in this research area were conducted under acute hypoxic conditions. Umezu *et al.* were the first to report intercellular communication via exosome under chronic hypoxia[93]. A few hypoxia-resistant multiple myeloma (MM) cell lines were developed after incubation in hypoxic conditions for more than six months to mimic the hypoxic bone marrow environment *in vivo*[93]. Increased exosomal level of miR-135b was detected in these hypoxia-resistant MM cells, and it was shown to promote endothelial tube formation under hypoxia via the HIF-FIH signaling pathway[93].

Promotion of cancer cell invasion and metastasis by hypoxic tumor-derived exosomes

Exosomal ncRNAs within the hypoxic TME are known to regulate tumor invasion and metastasis by modulating EMT. In breast cancer, higher expression of miR-25-3p in hypoxic cancer-derived exosomes was found to stimulate cancer proliferation and migration by inducing IL-6 secretion and activating NF-κB signaling in macrophages[94]. In bladder cancer, the lncRNA (UCA1) preferentially secreted by hypoxic cancer cells was shown to promote cancer growth by stimulating EMT both *in vitro* and *in vivo*[95]. In oral squamous cell carcinoma, a higher level of miR-21 was detected in the exosomes from hypoxic cancer than those from normoxic cancer to promote migration and invasion by inducing EMT[96]. The interaction between stromal and cancer cells via exosome within the TME plays a critical role in the initiation of metastasis. Lung cancer cells have been shown to take up exosomes secreted from hypoxic bone marrow-derived mesenchymal stem cells (BMSCs) and acquire a greater tendency for invasion[97]. Three miRNAs (miR-193-3p, miR-210-3p, and miR-5100) showing a high abundance in hypoxic BMSC-derived exosomes were transferred to cancer cells and subsequently activated STAT3 signaling to induce EMT in the lung cancer cells[97]. Interestingly, these three miRNAs were also found to be upregulated in plasma-derived exosomes from lung cancer patients with metastatic disease than in non-metastatic patients[97].

Modulation of cancer immune system by hypoxic tumor-derived exosomes

Reduced immune surveillance is the major reason allowing primary tumors to develop metastasis in distant secondary organs[98,99]. Tumor-secreted exosomes have been reported to induce T-cell apoptosis, inhibit interferon gamma-dependent expression of macrophages, suppress natural killer (NK) cell activity, and increase myeloid-derived suppressor cell (MSDCs) population, which collectively suppress immune surveillance and allow tumor growth[100,101]. Figure 2 illustrates the major mechanisms by which hypoxic tumor-derived exosomes promote an immunosuppressive TME.

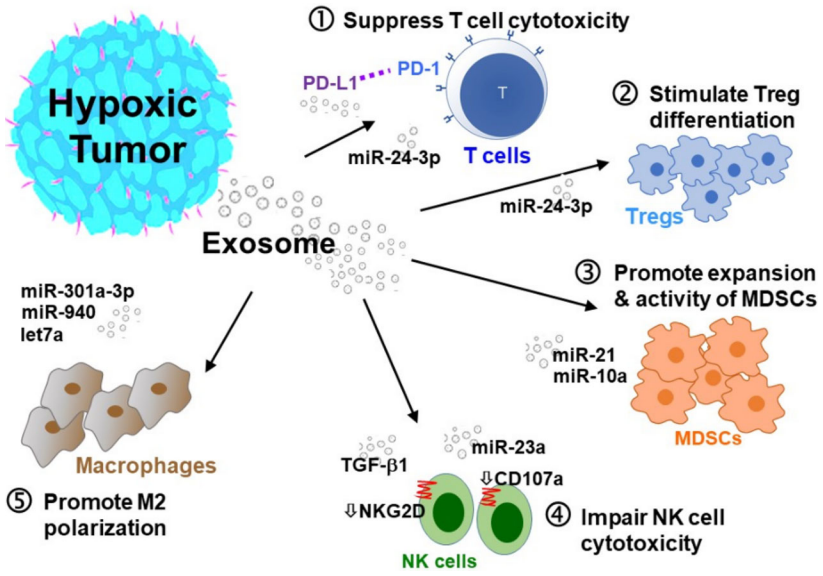

Figure 2. Hypoxic tumor-secreted exosomes promote an immunosuppressive TME. Hypoxic tumor-secreted exosomes promote an immunosuppressive TME by interfering with several intracellular pathways and modulating immune accessory cells, including cytotoxic T cells, T-regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), natural killer cells (NK), and tumor-associated macrophages (TAMs). (1) Inhibition of T cell proliferation; (2) stimulation of Treg differentiation; (3) induction of MDSCs; (4) impairment of NK cells; and (5) stimulation of M2 polarization of TAMs.

Inhibition of T cell proliferation

Tumoral exosomes loaded with biologically active cargoes have been proposed to mediate the intercellular transmission of signals within the TME to promote immune escape and tumor progression. Ye *et al.* were the first to report a differential miRNA signature from nasopharyngeal carcinoma-derived exosomes to mediate T cell dysfunction[102]. The induction of exosomal miR-24-3p in nasopharyngeal carcinoma-derived exosomes under hypoxia was found to inhibit T cell proliferation but promote differentiation of T-regulatory cells (Tregs) by targeting FGF11 via the upregulation of p-ERK, p-STAT1, and p-STAT3 and downregulation of p-STAT5[103].

Immune checkpoint inhibitors including anti-programmed cell death receptor (PD-1) (nivolumab and pembrolizumab) or anti-PD-ligand (PD-L1) (duralumab, atezolizumab, and avelumab) monoclonal antibodies are revolutionizing cancer therapy. They lead to durable anticancer responses and overall survival benefits in a wide range of cancer types[104]. PD-1 is an inhibitory receptor expressed on activated T cells, B cells, and natural killer cells, which blunt the immune response under physiological conditions. The T cell-mediated cancer-killing effect will be suppressed when PD-1 is occupied by its major ligand PD-L1 (expressed in tumor cells and infiltrating immune cells). Anti-PD-1/PD-L1 antibodies work by binding to the inhibitory PD-1 receptors on tumor-reactive T cells and PD-L1 on tumor cells, respectively, to disrupt the PD-1/PD-L1 interaction and reactivate the cytotoxic T cell activity.

Despite the breakthrough of anti-PD-1/PD-L1 immunotherapy, the response rate is low. Moreover, most patients who initially respond to immunotherapy will eventually relapse because of adaptive resistance. To maximize the full potential of anti-PD-1/PD-L1 immunotherapy, the mechanisms underlying these *de novo* and adaptive resistance mechanisms is a research area of intensive investigation. When T cells recognize the tumor antigen on the cancer surface, they release interferons to induce PD-L1 expression in cancer cells[105]. The increased PD-L1 expression in cancer cells will then lead to specific inhibition of T cell recognition of cancer, subsequently resulting in a phenomenon known as adaptive immune resistance and inhibiting the

antitumor immune response. To this end, PD-L1 loaded in exosomes was shown to interact directly with T cells to suppress anticancer efficacy of chemotherapy in various cancer types, including breast[106], gastric[107] head and neck[108], melanoma[109], pancreatic[110], and prostate[111] cancer. It will be useful to elucidate whether hypoxia regulates the loading of PD-L1 into tumor-derived exosomes.

Induction of MDSCs

MDSCs are a heterogeneous population of immune cells from the myeloid lineage which migrate to tumor sites to create an immunosuppressive TME[112]. MDSCs suppress adaptive and innate immunity by inhibiting T cell activation, promoting macrophage M2 polarization, inducing CAF differentiation, and inhibiting NK cell cytotoxicity. The abundance of MDSCs at tumor sites is known to correlate closely with poor clinical prognosis and reduce the efficacy of immunotherapy in cancer patients. In glioma, miR-21 and miR-10a secreted in tumor-derived exosomes under hypoxia have been reported to promote the expansion and activity of MDSCs *in vitro* and *in vivo* via the miR-21/PTEN/PI3K/AKT and miR-10a/RORA/IkBα/NF-κB pathways, respectively[113]. Therefore, novel strategies to modulate hypoxic tumor-secreted exosomes may be developed to regulate MDSCs and potentiate immunotherapy[114]. To this end, miR-21 loaded in γδ T cell-secreted exosomes has been shown to abate the function of MDSCs by targeting PTEN in a PD-L1-dependent manner[115].

Impairment of natural killer cells

Hypoxia is also known to promote an immunosuppressive TME by attenuating cytotoxic T cell and Impairment of natural killer (NK) cell-mediated tumor cell lysis. Berchem *et al.* were the first to report the secretion of non-coding RNAs in exosomes from hypoxic lung cancer cells to impair NK cell cytotoxicity[116]. Under hypoxic conditions, higher miR-23a expression was observed in lung cancer cell-derived exosomes, which impaired NK cell cytotoxicity by targeting CD107a[116]. Moreover, the hypoxic tumor-derived exosomes were also shown to transfer TGF-β1 to NK cells, thereby reducing the cell surface expression of the activating receptor NKG2D and inhibiting NK cell cytotoxicity[116].

Stimulation of M2 polarization of tumor-associated macrophages

TAMs refer to the major tumor-infiltrating immune cells, which interact with the tumors and tumor-associated macrophages (TME) to regulate tumor immunity[117]. Macrophage polarization is the process by which macrophages adopt distinct functional phenotypes in response to environmental stimuli and signals. M1 macrophages are functionally pro-inflammatory and antimicrobial, whereas M2 macrophages are anti-inflammatory. M1 and M2 macrophages exhibit a high degree of plasticity and are converted into each other upon changes within the TME or anticancer therapies. Under hypoxic pressure, tumor-derived exosomes have been shown to induce M2 polarization in various cancer types. In pancreatic cancer, miR-301a-3p was highly expressed in hypoxic cancer cell-derived exosomes, and it was shown to promote macrophage M2 polarization by activating PTEN/PI3Kγ signaling pathway[118]. Coculture of pancreatic cancer cells with the hypoxic cancer-derived exosomes or miR-301a-3p-upregulated macrophages was shown to facilitate the epithelial-mesenchymal transition and lung metastasis[118]. In epithelial ovarian cancer (EOC), hypoxic tumor-derived exosomes were shown to express a high level of miR-940, and they stimulated M2 polarization of macrophages and promoted cancer proliferation and migration[119]. A differential miRNA expression signature was also identified in the EOC-derived exosome under hypoxia to promote M2 polarization. miR-21-3p, miR-125b-5p, and miR-181d-5p were induced by HIF-1α and HIF-2α in the exosomes under hypoxic conditions, which regulate SOCS4/5-STAT3 signaling to stimulate M2 polarization and a malignant TME[120]. In melanoma, let-7a was shown to be downregulated in hypoxic cancer cells but remarkably increased in the hypoxic cancer-derived exosomes[121]. The exosomes carrying let-7a were found to promote a metabolic shift towards enhanced mitochondrial oxidative phosphorylation in macrophages by suppressing insulin-Akt-mTOR signaling to enhance cancer progression[121].

SUMMARY

Rapidly expanding and hypoxic tumors exploit exosomes to communicate with both cancerous and non-cancerous cells in the TME to promote cancer survival and resist immune surveillance. Hypoxia has been shown to directly induce the production of exosomes, modulate the exosome cargo sorting process, and promote exosome uptake by recipient cells. Under low oxygen tension, cancer cells are primed to glycolytic metabolism, thus inducing an acidic TME to indirectly promote intracellular transport of the exosome. Numerous regulatory molecules are involved in the regulation of exosome biology under hypoxia. More studies are warranted to fully unravel the effect of hypoxia on exosome-mediated intercellular communication within the TME.

FUTURE PERSPECTIVES

Advances in precision oncology have led to the increasing application of tissue and liquid biopsy methods in clinical practice to facilitate treatment selection and monitoring of cancer progression. For the traditional method using tissue biopsy, limited tissue specimens are taken from the patients. They are not able to reflect the spatial and temporal heterogeneity of a primary tumor or between multiple potentially discordant metastatic lesions. In comparison with tumor tissue analysis, liquid biopsy is less invasive, and the samples can be obtained throughout disease progression. The liquid biopsy analytes include circulating tumor cells, circulating nucleic acids[122], extracellular vesicles[123], and other tumor-derived materials present in blood and other body fluids. Among various liquid biopsy analytes, exosomes are unique in the way that they contain not only DNA but also RNAs, ncRNAs, proteins, glycoconjugates, and lipids, thus making them more versatile biomarkers.

Currently, the exogenous hypoxic marker drug pimonidazole has been used to visualize hypoxic regions in histological sections of tumors in pathological research *in vivo*[124]. However, the method is invasive and involves the surgical removal of tumors for imaging. Therefore, the application of tumor-derived exosomes from biological fluid to reveal the presence of hypoxic tumors will be beneficial. A few exosome biomarkers have been shown to reflect the hypoxic status of tumors as well as the stage of tumor progression. Exosomes derived from hypoxic glioma cells were enriched with hypoxia-related mRNA and proteins (including caveolin 1, IL-8, MMPs, and PDGF)[34]. Importantly, patients presenting with high levels of these biomarkers were associated with worse survival[34]. In rectal cancer patients, low levels of miR-486-5p and miR-181a-5p but high levels of miR-30d-5p in the exosomes harvested from plasma samples are associated with hypoxic tumors and poor prognosis[125]. Indeed, exosomes have been used as diagnostic or prognostic tools for assessing hypoxic tumors in recent clinical trials [Table 3].

As hypoxic tumors produce exosomes to promote tumorigenesis, the inhibition of exosome formation and secretion may be exploited as a novel strategy to suppress tumor development. In an excellent recent review, He *et al.* summarized the strategies for exosomal targeting and discussed the potential clinical applications[126]. Experimental reagents such as manumycin A and GW4869 were shown to inhibit exosome biogenesis and secretion from mammalian cells[127]. On the other hand, the Rab family of GTPases involved in exosome secretion can also be targeted to hinder exosome-mediated intercellular communication. For example, Rab5a is involved in the early step of exosome biogenesis, whereas Rab11, Rab27a, and Rab35 regulate the fusion of multivesicular bodies with the plasma membrane and exosome secretion[128]. Downregulation of Rab27a has been shown to inhibit exosome-dependent and -independent tumor cell growth[128]. Specific inhibition of sphingomyelinase (an enzyme catalyzing the formation of ceramide from sphingomyelin) has also been shown to suppress exosome biogenesis and cargo loading, thereby retarding tumor growth[63].

Table 3. Representative clinical trials exploiting exosome biomarkers to assess hypoxic tumors

Cancer type	ClinicalTrials.gov Identifier	Aim(s) relevant to exosome biology	Current status
Lung cancer	NCT04629079 (LungExoDETECT)	• To validate exosomal assays that are based on hypoxia detection as potential biomarkers for early detection • Compare exosomal analysis with the standard of care imaging	Recruiting; Started in October 2020
Lung cancer	NCT04529915	• Multicenter clinical research for early diagnosis of lung cancer using exosomes derived from blood plasma	Active; Not recruiting; Started in April 2020
Colorectal cancer	NCT04394572 (EXOSCOL01)	• To identify new diagnostic protein markers (e.g., integrins and metalloproteases) for colorectal cancer in circulating tumor exosomes	Recruiting; Started in January 2021
Clear cell renal carcinoma	NCT04053855	• To analyze urinary exosomes as a liquid biopsy tool for early diagnosis of clear cell renal cell carcinoma	Recruiting; Started in August 2019
Ovarian cancer	NCT03738319 (EOC-EXOSOME)	• To analyze the expression of miRNA and lncRNA from exosomes in blood samples by next-generation sequencing in patients with high grade serous ovarian cancer or benign gynecologic diseases	Recruiting; Started in November 2018
Melanoma	NCT02310451	• Pilot study to examine exosomes collected from the blood before and after BRAF inhibitor therapy in patients with advanced unresectable or metastatic BRAF mutation-positive melanoma • To develop an exosome-based theranostic tool for personalized care in melanoma patients	Recruiting; Started in 2016

miRNA: MicroRNA; lncRNA: long non-coding RNA.

The removal of oncogenic exosomes has been investigated as a novel therapeutic strategy for cancer therapy[129]. Mesoporous silica nanoparticles loaded with EGFR-targeting aptamers have been used to mop up circulating cancer-secreted EGFR+ exosomes, thus preventing their entry into the small intestine to suppress metastasis of lung cancer cells[129]. On the other hand, exosomes from immune cells were shown to exhibit anticancer activity[17]. Recently, Jiang *et al.* reported the induction of exosome production from NK cells under hypoxia[130]. More importantly, compared to normoxic conditions, NK cell-derived exosomes were found to express remarkably higher levels of FasL, perforin, and granzyme B in hypoxia to produce a higher NK cell cytotoxic effect[130]. Therefore, hypoxia-treated NK cells may be used to potentiate cancer immunotherapy.

Exosomes may also be employed as a drug delivery system for cancer therapy. Drugs or therapeutic siRNAs could be loaded into exosomes by various methods such as direct incubation, electroporation, and sonication[131]. Zhuang *et al.* reported the encapsulation of curcumin or an investigational STAT3 inhibitor (JSI124) in cancer cell-derived exosomes by incubating the exosomes with the drugs[132]. The drug-loaded exosomes were delivered to the brain for the treatment of inflammation via an intranasal route[132]. Besides, therapeutic siRNAs could also be loaded into the hydrophilic core of exosomes in the pharmaceutically active form[133,134]. Recently, Alvarez-Erviti *et al.* reported the successful delivery of siRNAs to the mouse brain using dendritic cell-derived exosomes[135]. Alternatively, the anticancer drug paclitaxel has been loaded indirectly into exosomes secreted from gingival mesenchymal stromal cells (MSCs) after co-culturing them with the drug[136]. Importantly, the exosomes derived from MSCs after priming with paclitaxel were shown to exhibit significant anticancer activity against human pancreatic cancer cells *in vitro*[136]. Interestingly, in a recent study examining anticancer drug delivery by exosomes, exosomes from hypoxic human breast cancer cells loaded with olaparib (a PARP inhibitor) were found to exhibit a superior uptake rate when they were co-cultured with hypoxic cancer cells[20]. A more detailed investigation of exosome loading and production under hypoxic conditions is advocated to further optimize exosome-mediated drug delivery.

DECLARATIONS

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Authors' contributions

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Cancer Drug Resistance

Original Article

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Electrochemotherapy using thin-needle electrode improves recovery in feline nasal planum squamous cell carcinoma - a translational model

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Abstract

Aim: Cutaneous squamous cell carcinoma (cSCC) is a common disease in patients exposed to UV-light and human papillomavirus. Electrochemotherapy, a well-established treatment modality with minimum side effects in human and veterinary medicine, circumvents chemoresistance to bleomycin by the use of electric fields. However, patients are sensitive to the trauma produced by the insertion of the needles that lengthen recovery times, particularly cats with nasal planum cSCC. To address this matter, we developed thin-needles electrodes.

Methods: Thin-needles electrodes developed using computer simulations and plant tissue models were compared to standard electrodes. A prospective non-randomized study recruiting 52 feline patients with nasal planum cSCC was performed. Local response, anorexia, and overall survival were evaluated.

Results: Computer simulations and plant model experiments showed satisfactory results with both electrodes. The patients treated with the thin-needle electrode obtained similar local response rates compared to the standard group, OR 97.3% *vs.* 80%, respectively ($P < 0.067$). Most patients in the thin-needle group resumed eating in less than 48 h, as the anorexia was significantly lower ($P < 0.0001$). Using the standard electrode, most patients took 3 to 5 days to resume normal feeding. The electric current circulating in the standard electrode was 44% higher, contributing to a longer duration of anorexia due to tissue damage. The overall survival in both groups was similar.

Conclusion: Electrochemotherapy using thin-needle electrodes provides equivalent local response rates and overall survival compared with standard electrodes but significantly reduced return to appetite after the treatment. These results may be useful in the development of new electrodes for human patients.

Keywords: Cancer, SCC, nose, cat, electroporation, ECT, tumor, cSCC

INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a malignant neoplasm of keratinocytes of the skin. It is one of the most common malignancies diagnosed in humans. Older people are at an increased risk, with a ten-fold increase in patients above 70 years. People with light skin color, light-colored eyes, and light hair, who are predisposed to sunburn, are at an increased risk. In this context, UV light exposure acts as a tumor-initiating and tumor-promoting factor by inducing the formation of actinic keratosis, a precursor lesion of cSCC. The mutation of both *p*53 genes is an early event in carcinogenesis, and it is the most common mutation found, but not the only one[1]. An association between human papillomavirus (HPV) and cSCC has been reported[2].

The similarities between human cSCC and feline cSCC are remarkable. In cats, this tumor mostly occurs in areas exposed to UV light. Particularly, areas without fur, such as the nasal planum, the pinnae, and the eyelids, are at greater risk. As the damage to the skin is cumulative, the cancer mainly affects cats older than 10 years, which according to their lifespan, is equivalent to humans around 70 years of age. Typically, a history of chronic solar exposure is present, where actinic changes lead to carcinoma *in situ* (noninvasive carcinoma confined to the epidermis, similar to the actinic keratosis in human patients), which progresses to invasive carcinoma. White-haired cats have an increased risk of developing cSCC than other hair colors as they lack the protection of melanin. More than half of the cats with nasal planum cSCC have mutations of the tumor-suppressor gene p53, indicating that a genetic predisposing factor may have an important role in the development of the disease[3-5]. Interestingly, HPV has been isolated from cSCC in cats, supporting that it can contribute in the same way to the disease development[6]. Resistance to chemotherapy and failure of combined treatments (radiotherapy plus chemotherapy) is a concern, which leads to a poor prognosis among affected patients[7-12]. Due to the similarities between the cSCC of cats and humans, cats provide a very good translational model for improving or developing new treatment approaches[13,14].

Diagnosis

Physical examination, including evaluation of mandibular lymph nodes, is standard. The definitive diagnosis is made with histopathology. For complete staging, the presence of metastasis in the nodes should be assessed with cytology or biopsy. Full biochemical panels, three-view thoracic radiographs, and complete blood cell counts are advised before treatment[15].

Treatment options

The preferred treatment for cSCC is surgery when possible and accepted by the owner. Wide surgical resection provides long-term control of the disease in most situations[16]. Another effective treatment option

is radiotherapy (RT), which provides good results with mild self-limiting side effects for patients at the initial stages of the disease[17]. RT may also be combined with surgery to improve treatment outcomes, even with incomplete resections[18]. When the lesion is small, up to 0.5 cm in diameter, cryosurgery is a treatment option that may be used successfully. However, many sessions may be required for adequate control of the disease[19]. Similarly, photodynamic therapy (PDT) is another emerging treatment option. In one study, 90% of the treated lesions achieved a complete response, but 60% of them relapsed. As with cryotherapy, PDT may require many sessions, and it is most suitable for superficial lesions[20].

There is limited evidence of the use of chemotherapy for this disease. The use of carboplatin with sterile sesame oil injected into the nasal planum showed modest results, with a complete response rate of 73% and 55% progression-free survival at one year[21]. The exposure of the medical staff to chemotherapy during treatment is no longer acceptable in the interest of safety, and for that reason, safety measures for handling chemotherapy are mandatory. Among effective chemotherapeutic drugs for treating cSCC, bleomycin is a good choice. Bleomycin, an antibiotic with anticancer properties, was discovered by Umezawa, who published its anticancer effects in 1966. He described its curative effect on skin cancer in dogs. The dog and mouse were the first translational models for this drug[22]. One of the first uses of bleomycin in human patients was in the treatment of cSCC, where the minimum effective dose was 12 cycles of 6,000 IU/m^2. Considering the average body surface area for a human patient is around 2 m^2, a total dose of 144,000 IU was used. The treatment could be continued after the 12th dose if good results were observed[23]. Other studies report using doses twice as high, achieving good local responses in cSCC, but with significant pulmonary and skin complications[24]. Bleomycin is non-permeant to the cell membrane, and to be internalized, it has to interact with specific membrane proteins[25,26]. The sensitivity of tumors to bleomycin depends on the presence of these proteins in the cell membrane, as well as the concentration of an enzyme called bleomycin hydrolase capable of degrading it[26]. Typically, the skin and lungs have very low concentrations of this enzyme, explaining their sensitivity to it[27]. The drug was approved by the FDA in 1975 for the treatment of cSCC[28]; however, its use steadily declined over the years due to its serious side effects when cumulative doses were high. Today, bleomycin is still used in combination with other agents for the palliative treatment of cSCC in human patients, with a cumulative dose capped at 400,000 IU[29]. In this context, with the discovery of electrochemotherapy by Mir *et al.* in 1997, the resistance mechanisms were overcome and the drug could once again be used with greatly reduced doses, increasing its effectiveness and reducing its side effects[26,30].

Electrochemotherapy

Electrochemotherapy (ECT) is a well-established treatment modality in veterinary medicine for treating cutaneous, subcutaneous, and mucosal tumors[31-34]. An aspect of ECT that sometimes is difficult to understand is how a tumor previously resistant to bleomycin can become exquisitely sensitive to it, even to the point of drastically reducing doses and still obtaining very good results.

Electroporation, depending on pulse parameters, can be irreversible, where the cells affected by the electric field die by necrosis, or reversible, where the affected cells are spared. When an electroporation-based treatment is performed, there is always an extent of reversible and irreversible electroporation coexisting[35].

In reversible electroporation, cell membrane permeabilization is transient, permitting the introduction of certain molecules into the cytosol, such as bleomycin[35]. The combination of reversible electroporation with bleomycin or cisplatin is termed ECT. Once inside the cell, bleomycin cuts DNA strands and impedes mitosis of cells inducing mitotic cell death. The treatment displays selectivity towards dividing cells, sparing quiescent ones. Then, normal cells have time to proliferate and repair tissue defects, allowing excellent

cosmetic results. For these reasons, ECT provides very good local response rates with minimum side effects[36].

ECT may be used independently or in combination with surgery, especially when the surgery cannot provide clean margins. ECT may be used as a neoadjuvant, adjuvant, or concomitant therapy[37-40]. Some authors have reported an objective response rate of 97.3% when treating feline patients with nasal planum cSCC with minimum side effects[41]. ECT may also be used in combination with chemotherapy, RT, or cryosurgery for selected cases[40]. In the Discussion Section, we present our recommendations for performing ECT alone or combined with other therapies for treating cSCC in cats.

As mentioned above, it is reported in the literature that ECT has minor and self-limited side effects, along with very good clinical responses[42]. However, when treating the nasal planum in feline patients, we observed increased sensitivity to the trauma produced by the insertion of the needles. The lesions produced lead to subsequent anorexia that sometimes requires supportive treatment[43,44]. To evaluate a role for less trauma, our group developed two electrodes: (1) the first, labeled standard, is very similar to commonly used electrodes in standard ECT; and (2) a thin-needle electrode with needles of a reduced diameter. We evaluated electric field distribution and electroporated area to determine if the new design (2) would provide an adequate electric field distribution. A poorly designed electrode can lead to local recurrence since the tumor can be left insufficiently treated[45]. In the patients, we evaluated response rates, the severity of anorexia, and overall survival times to determine if the new electrode would improve the effectiveness of the treatment and the post-procedure anorexia.

METHODS

Two electrodes were used in this study: (1) the standard electrode, composed of four 20 G needles arranged in two rows, 4 mm apart; and (2) the thin-needle electrode with the same geometry but with four 25 G needles. Both electrodes use disposable needles [Figure 1A]. The configuration of the electroporator was the same for both types of electrodes [Figure 1B].

To assess the electric field distribution, both electrodes were simulated in COMSOL Multiphysics 4.3, applying a voltage-to-distance ratio of 1000 V/cm, according to the updated standard operating procedures for ECT[46].

Electrical resistance from tumors was measured using the standard electrode and used in the COMSOL model. Afterward, the design of the electrodes was validated in a simple plant model[47]. With the final design, the two electrodes were fabricated, and the selected patients were treated [Figure 1C and D].

Patient selection and group allocation

Fifty-two feline patients were selected for treatment based on confirmed histopathology and nasal planum location. Cats were randomly assigned to one of the experimental groups [standard needles (1) or thin-needle group (2)] in an alternating pattern. When 15 cats were placed in the standard group (1), we added the remaining cats only to the thin-needle group (2). A full physical examination was performed, along with complete blood work (CBC, chemistry, and UA) to assess the status of the patient and the anesthetic risk. The patients were staged following the WHO criteria[48] [Table 1]. In addition, feline immunodeficiency virus (FIV) was tested in all the patients and correlated with the results.

Follow-up

Follow-up visits were scheduled every two weeks until the final response was obtained (a complete response

Table 1. Staging system for feline cSCC (WHO criteria)[48]

Grade	Description
T0	No evidence of tumor
Tis	Tumor *in situ*
T1	Tumor < 2 cm in diameter
T2	Tumor 2-5 cm diameter or minimally invasive
T3	Tumor > 5 cm diameter or with the invasion of subcutis
T4	Tumor invading other structures such as the fascia, muscle, or bone
N0	Absence of lymph node metastasis
N1	Presence of lymph node metastasis
M0	Absence of distant metastasis
M1	Presence of distant metastasis

As all patients were N0M0, the stages were defined as: Stage I = T1, Stage II = T2, Stage III = T3, and Stage IV = T4.

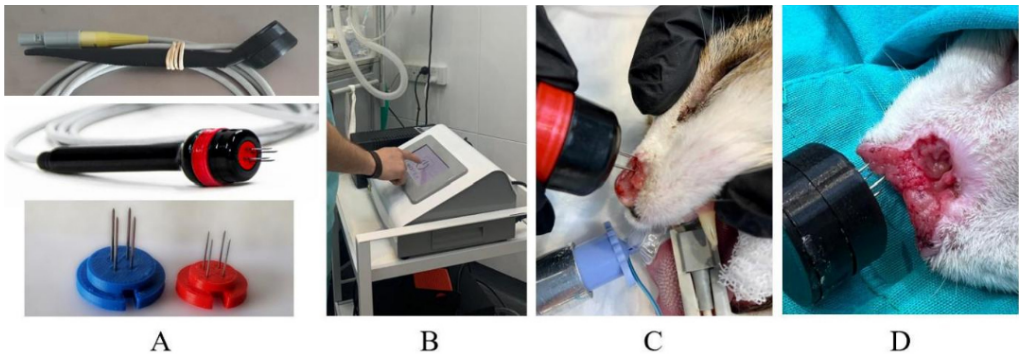

Figure 1. Images showing the electrodes and the electrochemotherapy procedure. (A) (top) the standard electrode; (middle) the thin-needle electrode; and (bottom) the disposables 20 G (left) and 25 G (right). (B) The operator is configuring the electroporator. (C) A patient is treated with the thin-needle electrode. (D) A patient is treated with the standard electrode.

or a lesion stable in size which was not growing back again) and monthly thereafter. We evaluated local response using modified RECIST 1.1 criteria for solid tumors[49].

Complete response (CR) was defined as complete eradication of the treated tumor with re-epithelialization of skin.

Partial response (PR) was defined as a decrease of > 30% in the sum of the diameters of measurable lesions.

Stable Disease (SD) was defined as a reduction of < 30% or an increase of < 20% of the above-mentioned measurements.

Progressive disease (PD) was defined by an increase of > 20% of the above-mentioned measurements. Responses were confirmed one month after they were achieved.

Anorexia after the procedure was evaluated following the criteria of LeBlanc *et al.*[50], where anorexia is defined as "a disorder characterized by a loss of appetite/decreased interest in food". Table 2 presents the classification of anorexia.

Table 2. Grading of anorexia extracted from LeBlanc *et al.*[50]

Grade	Description
1	Complete anorexia lasting < 48 h
2	Complete anorexia lasting 2-3 days
3	Anorexia lasting 3-5 days; associated with significant weight loss (≥ 10%) or malnutrition; IV fluids, tube feeding, or force-feeding indicated
4	Life-threatening consequences; total parenteral nutrition indicated; complete anorexia lasting > 5 days
5	Death

The electric current that circulates during each pulse is shown on the screen of the electroporator. This allows us to monitor the treatment and seek a relation to the side effects.

Anesthetic procedure

Patients were treated under general anesthesia using intramuscular administration of xylazine (Xylazine 100®, Richmond, Buenos Aires, Argentina) 0.5 mg/kg and tramadol (Tramadol®, John Martin, Buenos Aires, Argentina) 2 mg/kg. Induction was performed with intravenous administration of propofol (Propofol Gemepe®, Gemepe, Buenos Aires, Argentina) 2-3 mg/kg. For maintenance, isoflurane (Zuflax®, Richmond, Buenos Aires, Argentina) 2%-3% and intravenous fentanyl (Fentanyl Gemepe®, Gemepe, Buenos Aires, Argentina) 2 μg/kg were used. Amoxicillin with clavulanic acid (Clavamox® Zoetis, Buenos Aires, Argentina) 15 mg/kg/bid and meloxicam (Meloxivet®, John Martin, Buenos Aires, Argentina) 0.2 mg/kg/sid were administered orally for prophylaxis and analgesia after the treatment according to the needs of each patient.

Electrochemotherapy procedure

Intravenous bleomycin was administered at 15,000 UI/m^2 body surface area. After 5-8 min, to allow drug distribution, the electric pulses were delivered[46] with a monopolar square-wave electroporator (EPV-200, BIOTEX SRL, Buenos Aires, Argentina). The pulse trains applied with both electrodes were eight 100 μs long pulses of 400 V (1000 V/cm) at a repetition frequency of 5 kHz[46].

Statistical analysis

Statistical analysis was made using MedCalc 14.8.1. To compare age, sex, and body weight, the independent samples t-test and ANOVA, respectively, were used. To compare the anorexia score, the Mann–Whitney test was used. The comparison of the response rates obtained utilized the Fisher's exact test. For survival comparison, Kaplan–Meier curves of survival were used, and the significance was assessed with the Log-Rank test.

RESULTS

COMSOL simulations showed that the electric field was distributed between the needles in both electrodes, which was above 480 V/cm. This electric field intensity was enough for reversibly permeabilizing the tissues. In addition, the area above 1050 V/cm was studied, which is the threshold for irreversible electroporation[51]. To obtain the best results, the area of reversible electroporation should be maximized (or at least cover the whole area between needles) and the area of irreversible electroporation should be kept to a minimum. This exploits the benefits of ECT, as irreversible electroporation damages the tissue without selectivity and produces necrosis that should be avoided[52]. As shown in Figure 2A and B, the area of reversible electroporation was adequate on both electrodes. However, the area of irreversible electroporation was larger in the standard electrode, thus contributing to the differences in trauma within this treatment group. The plant models correlated with the simulations [Figure 2C and D].

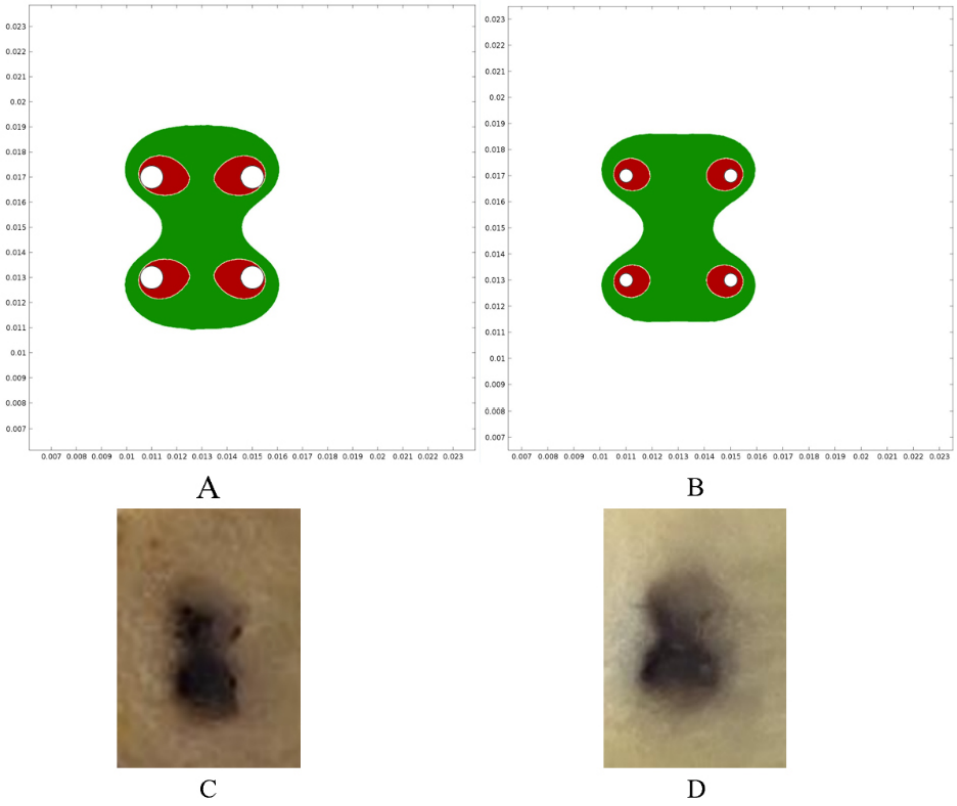

Figure 2. Electric field simulations and vegetable experiments. (A) Reversible and irreversible areas of permeabilization in the standard electrode. (B) The same areas in the thin-needle electrode. Green indicates the electric field was above the reversible electroporation threshold (480 V/cm) and below the irreversible electroporation threshold (1050 V/cm). Red indicates the area where the field was above the irreversible threshold (1050 V/cm). These areas were obtained using COMSOL simulations of the electric field distribution. As can be seen, in both electrodes, the region between needles is covered by reversible electroporation, but the standard electrode presents larger areas of irreversible electroporation. (C,D) The electroporated plant tissue showed an electroporated area very similar to the simulations: (C) the standard electrode (1) was used, and (D) the thin-needle electrode (2) was used.

The composition of the experimental groups is shown in Table 3. There were no statistically significant differences among groups regarding age (ANOVA, $P = 0.601$), sex (independent samples t-test, $P = 0.768$), or body weight (ANOVA, $P = 0.185$).

During follow-up, if a relapse was observed, i.e., the growth of a previously shrinking lesion, the patient was scheduled for a new treatment session. The average number of treatment sessions for the standard group (1) was 1.7 (median 2, range 1-5) and for the thin-needle group (2) was also 1.7 (median 2, range 1-3).

The response rate obtained in the standard group (1) was as follows: 40%CR, 40%PR, 13%SD, and no PD. The OR rate was 80%. For the thin-needle group (2), the results were 70.3%CR, 27%PR, 2.7%SD, and no PD. The OR rate was 97.3% [Figure 3]. There were no statistically significant differences between the responses obtained in the groups (Fisher's exact test, $P = 0.067$). The cosmetic results were very good. In some cases, small scars developed not because of the treatment but because the tumor had already invaded and damaged the tissues, and thus a second intention healing with a scar is seen after the tumor responded to the treatment [Figure 4].

Table 3. Composition of the experimental groups. The details of both experimental groups are presented. The differences in sex (independent samples t-test, *P* = 0.768), age (ANOVA, *P* = 0.601), and body weight (ANOVA, *P* = 0.185) were not significant between groups

	Standard	Thin-needle
n	15	37
males/females (*P* = 0.768)	7/8	16/21
FIV+	7	6
Average age [years] (*P* = 0.601)	11.3	11.1
Average body weight [kg] (*P* = 0.185)	4.78	3.92
Stage I	26.7%	43.2%
Stage II	26.7%	40.5%
Stage III	20.0%	13.5%
Stage IV	26.7%	2.7%

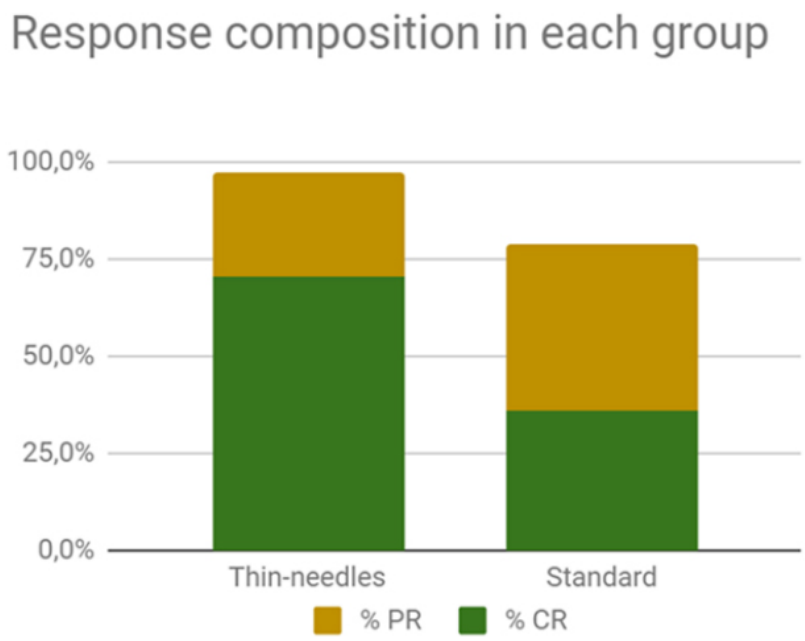

Figure 3. Composition of the objective response rates in each group (97.3% and 80% for the thin-needles and standard group, respectively). The differences between groups are not significant.

The anorexia score of the standard group (1) was, on average, 3.1 (median 3, range 1-5) and for the thin-needle electrode group (2) was 1.3 (median 1, range 1-3), the difference is statistically significant ($P < 0.0001$). These results show that thin needles significantly reduced post-treatment anorexia in the patients. Subjectively, the area treated looked much less inflamed after the treatment using the thin-needle electrode when compared with the standard electrode. No systemic toxicity was observed in either of the groups.

Measurements of the average electric current that circulated during the delivery of the pulses were 2.73 A in the standard group (1) and 1.89 A in the thin-needle group (2). This may be attributed to the larger surface contact with the standard needles. Approximately 44% more electric current circulated with each pulse when the standard electrode was used. The circulating electric current induces electrolysis of water molecules producing extreme pH changes in the region of the electrodes[53,54]. Even though they are transient, these changes can contribute to tissue damage and inflammation, and they may play a role in

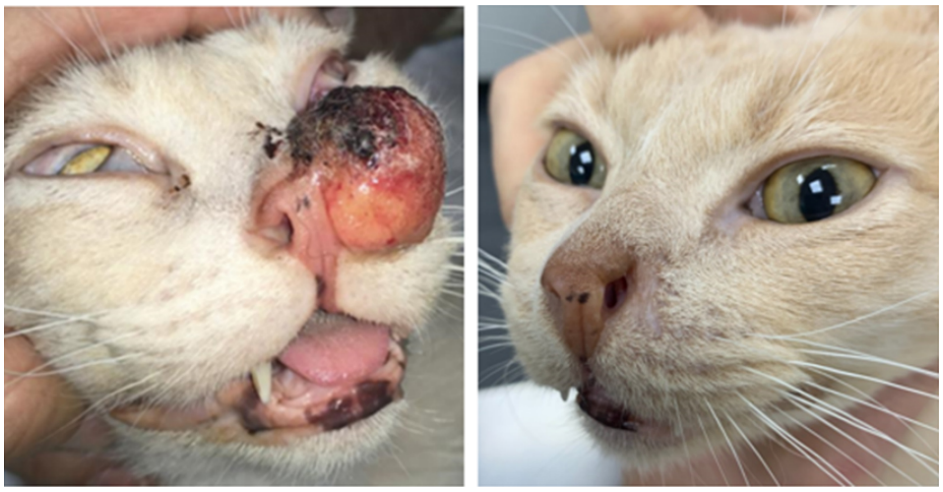

Figure 4. Feline patient with cSCC in the nasal planum treated with ECT using the thin-needle electrode: (left) before the treatment; and (right) 51 days after the treatment. The patient obtained CR. As can be seen, minimum tissue scarring is present where the tumor was located.

worsening anorexia after the treatment.

FIV+ patients showed more PR than CR when compared with the FIV- patients. This makes sense considering that ECT relies mainly on the immune system to produce a clinical response[31]. However, a specific study is needed to validate this observation.

The median overall survival for the patients in the thin-needle group (2) was 611 days (ranging from 170-1003 days), and for the standard electrode (1) was 520 days (ranging from 23-840 days) [Figure 5]. The differences were not statistically significant, supporting that using thin needles does not reduce the survival probability of the patients (Log-Rank test, $P = 0.019$). At the end of the study, 54% (20/37) of the patients in the thin-needle electrode group (2) remained alive, while 20% (3/15) were alive in the standard electrode group (1).

The Kaplan–Meier curves of survival by stage are shown in Figure 6A, where the differences between stages I and II as well as between III and IV were not statistically significant (Log-Rank test, $P = 0.179$ and $P = 0.113$, respectively). If we group stages I and II as early stages and III and IV as late stages, survival was significantly better at earlier stages (Log-Rank test, $P < 0.0001$), as shown in Figure 6B. This result was expected and is concurrent with the experience of other authors who have used ECT[42,43].

DISCUSSION

Companion animals are increasingly accepted models of human disease[55]. In oncology, translation from murine models only leads to approved drugs for human use in 11% of the cases[56], leading to the expenditure of billions of dollars[57]. The situation for companion animals as models is very different from murine models because of their unique characteristics. Companion animals work as sentinels for human diseases, due to their exposure to similar environmental hazards as their owners[58]. For example, the cSCC described in this work has the same risk factors and behavior as its human counterpart[59], with tumors developing in the context of complex tumor–stroma interactions[60] and in the presence of an intact immune system[61]. Both characteristics are very important for tumor resistance to different therapies present in human patients. In addition, the size of the animals allows multiple tissue sampling, and the same

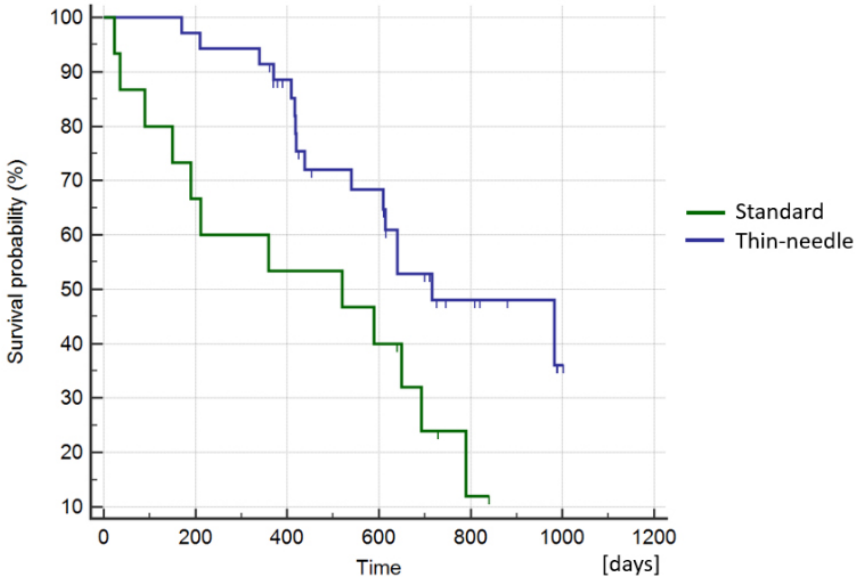

Figure 5. Kaplan-Meier curves of survival comparing the standard group *vs.* the thin-needle group. The curves show no statistically significant difference, meaning that the use of thin needles does not affect the long-term response (Log-Rank test, $P = 0.019$).

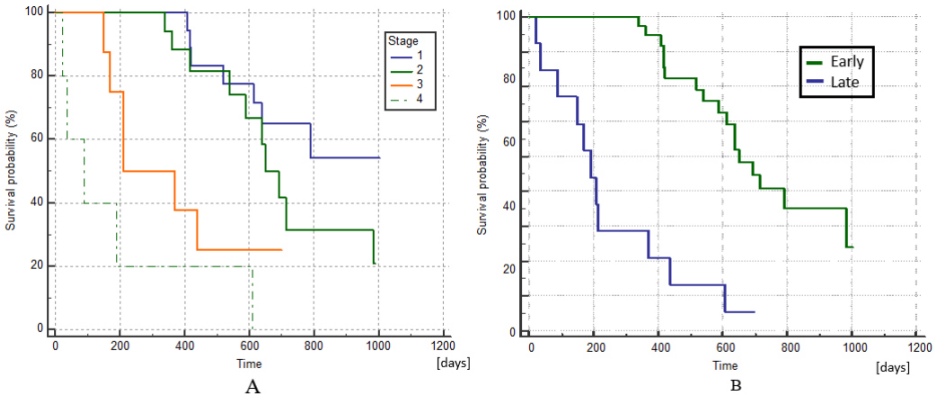

Figure 6. Kaplan-Meier curves of survival comparing patients at different stages of the disease. (A) There are no statistically significant differences in survival between stages I and II or between III and IV (Log-Rank test, $P = 0.179$ and $P = 0.113$, respectively). (B) Kaplan-Meier curves of survival comparing patients at early stages (I + II) with late stages (III + IV), which are significantly different (Log-Rank test, $P < 0.0001$).

procedures for diagnosis and treatment are used, making the translation more efficient. The lifespan of companion animals is short; thus, clinical trials can provide results in a reduced timeframe[55]. Another interesting point is that owners provide very valuable information regarding their pets because they know them and can perceive changes in appetite, mood, or behavior. Finally, from the ethical viewpoint, the animals used in the studies already have the disease, and if the treatment under investigation works, there is a benefit for the patient and the owner.

ECT is a very valuable therapy in our setting, in both human and veterinary medicine. In the case of tumors of the nasal planum in cats, we use the following approach for ECT. (1) As stand alone therapy is used when the tumor can be completely treated in one treatment session. This means a tumor around 3 cm^3, with an invasion depth of less than 2.5 cm (the length of the needles). (2) As cytoreductive therapy is used before

surgery to increase the chances of success of the latter. (3) ECT may also be used during surgery, after the removal of the tumor to clean the tumor bed and reduce the risk of recurrence. In this case, plate electrodes are very useful since the tumor is removed, and the margins do not need to be too deep in the tissue. In addition, ECT may be used for treating relapses after surgery or RT. We reserve RT for very large tumors when the risk of relapse with a single ECT session is high. Very large tumors can be challenging to treat with ECT, as areas may be left untreated unintentionally[40]. If large tumors are meant to be treated with ECT, a close follow-up is advisable. During the follow-up, if a regrowth inside the treated area is observed, a new treatment session should be scheduled quickly. We do not perform ECT sessions on a fixed-time basis; when repeating the ECT session, a relapse is the reason after a complete remission[42]. In some cases, where a relapse inside the treated area is small, cryosurgery can be performed. In our experience, this is less costly for the owner than other ECT, and, for small relapses, it can be equally effective.

Human and veterinary practices of ECT share many similarities that allow us to take advantage of the results obtained from both human and veterinary patients. However, there are many differences among them, as in veterinary medicine, the variety of patients is wider, and their characteristics greatly differ. Treating the back of a Doberman, where very sharp and rigid needles are needed, is different from treating the sensitive nose of the cat. Veterinary ECT is more challenging if we consider the variety of species that can be treated, i.e., cats[37,39,62], dogs[63,64], horses[65,66], and even wild animals[67,68], and, ideally, an equal variety of electrodes should be available. The inconveniences of using the same electrodes for every patient are often accepted, but it may lead to poorer results for the patients. This challenge is not present in human medicine, since the human skin is similar among different subjects.

To address this concern and add options to the available list of electrodes for ECT, we developed a thin-needle electrode. To validate the design and study possible problems with the available electrodes, we performed simulations in COMSOL. These simulations showed that an important area of irreversible electroporation was produced when using the standard needles. This area is not negligible and should be considered in the treatment planning. Irreversible electroporation is used mainly for the treatment of visceral neoplasia, and not used in the skin, as necrosis will impact healing[69-71]. This delay in the healing process may have contributed to additional days of anorexia in the patients treated in the standard group. In previous work, we demonstrated the presence of extreme pH fronts emerging from the needles during the delivery of the pulses[53]. Even if they are rapidly neutralized by the tissue buffers, extreme pH conditions surrounding the electrodes add damage to the irreversibly electroporated area. Damaging the tumor with these extreme pH fronts may not be important, but damaging healthy tissue is. Reducing the diameter of the needles will reduce their contact surface and the circulating current (44% less electric current), thus reducing the extension of the pH fronts and thus tissue damage[53]. Regarding human patients, the use of thin-needle electrodes may reduce inflammation and can improve patient comfort after treatment. It may allow the reduction of medication for pain management and improve patient acceptance to return for additional treatment if needed.

As was expected, there was no statistically significant difference between the local response rate using both electrodes (Fisher's exact test, $P = 0.067$) or in the survival time of the patients (Log-Rank test, $P = 0.019$). This is very important since ECT is an effective treatment, and any modification to the electrodes could negatively affect its performance. Even if it was not significant, a better response rate was seen in the thin-needle group, which may be due to the higher proportion of early-stage (I + II) tumors (53.4% in the standard needle group *vs.* 83.7% in the thin-needle group).

CRs were obtained only in patients at the early stages of the disease, with the early stage a factor significantly associated with the response (Fisher's exact test, $P < 0.0001$). Both results are in accordance with the results of other studies[43,72]. The microscopic bone involvement in later stages may act as a sanctuary for tumor cells[73]. The heterogeneity between soft tissues and bone leads to electric field inhomogeneity in the interface, reducing treatment effectiveness[42,74]. In addition, the bone may present with a reduced concentration of bleomycin due to its anatomical and physiological characteristics. To address this, a combination of intravenous plus intratumoral bleomycin has been proposed, allowing an optimal concentration of the drug in the poorly vascularized areas[75,76].

Surgery with clean margins can provide a median survival time of 360-594 days or more[16,77]. Photodynamic therapy showed a median time to recurrence of 133-392 days[78]. Cryosurgery can provide a median disease-free time of 270 days; however, only early-stage tumors (T1 or T2) can be successfully treated[79]. When using ECT as a single therapy, other authors have reported an overall survival time of 210-1260 days, being the survival longer in early-stage tumors[43,72]. Our results with ECT are in agreement with other ECT users and seem to be comparable to surgery with clean margins. It is very encouraging, as surgery is the treatment of choice because of the unavailability of electroporation worldwide compared to surgical options. Further study is needed to compare survival times of ECT with surgery to assess if ECT can be used as first-line therapy.

Regarding the adverse effects, Spugnini *et al.* used an electrode similar to our standard electrode, with needles of 1 mm in diameter[44]. They observed electrode-induced burns and scars, and some of the patients also experienced damage to the underlying tissues. These adverse effects were resolved in 2-3 weeks. The authors used a biphasic device, and, for that reason, the contribution of the pH to the damage was lessened. Denner S. Dos Anjos *et al.* identified local side effects; particularly, hyporexia was reported with a duration of seven days, including two cases where a feeding tube had to be placed for 7-14 days after the ECT procedure[43]. In this case, the authors used bipolar pulses too, but plate or needle electrodes were used depending on the size of the tumor. They reported using both in many cases. However, it is not clear if the hyporexia was worse when using plates, needles, or both at the same time. Again, even if the damage related to the extreme pH changes is reduced by the use of a bipolar device, the damage attributed to the irreversible electroporation cannot be ruled out. Using both electrodes in the same area may also contribute to increasing the area of irreversible electroporation by excessive overlapping of the electric field, i.e., overtreatment. It is also worth mentioning that all the complications were adequately managed, and the patients obtained good results. Tozon *et al.* did not report local or systemic adverse effects after ECT[72]; in this case, plate electrodes were used, and, thus, no trauma was produced. Using plate electrodes would be an option reserved for tumors with an invasion depth of a few millimeters[80]. Even though we did not use plate electrodes in this work, we used them for selected cases [Figure 7]. If the tumor invades deeper, then multiple sessions are needed when using plate electrodes. For that reason, invasive tumors may be treated better using needle electrodes.

Another important aspect where human medicine diverges from veterinary medicine is the mandatory use of disposable electrodes. In the latter, only a few manufacturers offer disposable electrodes, which in the authors' point of view, are very important for several reasons. First, new needles are sharp, and the tip is in optimal condition with each treatment. This significantly reduces the insertion trauma. Second, the conduction of the electricity is better, as there is no oxidation (which even occurs in stainless steel electrodes), providing an optimal electric field distribution. We strongly recommend using disposable needles in every case. In this study, their use was fundamental, as we kept the trauma induced by insertion to a minimum. Most patients underwent one or two treatment sessions (median 2). Spugnini *et al.* used

Figure 7. Feline patient with nasal cSCC treated with ECT using plate electrodes: (left) the patient during the treatment, where the plate electrodes were applied over the lesion; and (right) 54 days after the treatment, where a complete response was obtained.

more treatment sessions (median 4) to obtain similar objective response rates using caliper electrodes[81]. The fact that we needed fewer treatment sessions to achieve similar responses could be attributed to the use of disposable needles, which improve conductivity and produce a more homogeneous electric field distribution. However, in another study, Tozon *et al.*[72] reported performing mostly one treatment session and an 81.8% CR rate in 11 patients. In this case, the difference is that they used plate electrodes. Denner S. Dos Anjos *et al.* mostly performed one session but used plates and needles[43]. The combination may overcome the use of reusable needles at the expense of longer hyporexia time due to overtreatment. In any case, further study is needed to confirm that the use of disposable needles may reduce the number of treatment sessions.

In conclusion, cSCC is a common malignancy in feline patients exposed to UV light and HPV, which shares many characteristics with its human counterpart. For that reason, they are excellent models of human disease. The treatment of choice is surgery with clean margins, as other approaches are less effective. Particularly chemotherapy is not a good option because of cSCC's chemoresistance. However, ECT has overcome that barrier, being very effective. In particular, ECT using thin-needle electrodes in the nasal planum, when compared with standard electrodes, reduces anorexia times, improves recovery, and provides equally good results.

DECLARATIONS

Authors' contributions

Conceived the idea of the electrode, treated the patients, made the follow-up of the patients, analyzed the response: Tellado M

Built the prototypes of the electrodes, made experiments in plant tissue, treated the patients: Michinski S

Evaluating the results, writing and proofreading the manuscript: Impellizeri J

Worked in the computer simulations and in the models of the prototypes, analysed Computer simulations and correlated with experiments: Marshall G

General design of the study, analysed results and performed statistics: Signori E

Conceived the design of the electrodes, designed plant tissue experiments, treated the patients, analysed results, wrote the manuscript: Maglietti F

Availability of data and materials

Data is available upon request to the corresponding author.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethics approval and consent to participate

All regulations from the Consejo Profesional de Médicos Veterinarios (Argentina) were followed. Informed consents were signed by the owners. This work was approved by the IACUC of the School of Veterinary Sciences, University of Buenos Aires, Argentina. Protocol number: 2018/31.

Consent for publication

Consent from the owner to include the images of their pets was signed.

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Cancer Drug Resistance

Original Article

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Role of extracellular vesicles secretion in paclitaxel resistance of prostate cancer cells

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Abstract

Aim: The development of chemotherapy resistance is the major obstacle in the treatment of advanced prostate cancer (PCa). Extracellular vesicles (EVs) secretion plays a significant role among different mechanisms contributing to chemoresistance. Hence, inhibition of EVs release may increase the efficacy of chemotherapeutic drugs against PCa.

Methods: Paclitaxel (PTX) resistant PCa cells (PC3-R and DU145-R) were treated with GW4869, a known exosome biogenesis inhibitor. EVs were isolated from the conditioned media by ExoQuick-based precipitation method and characterized for concentration and size distribution by nanoparticle tracking analysis. The effect of GW4869 treatment on the survival and growth of PCa cells was assessed by MTT, and colony formation assays *in vitro*, and ectopic PC3-R xenografts in male athymic nude mice *in vivo*. The effect of other EV biogenesis inhibitors, imipramine and dimethyl amiloride (DMA), treatment was also analyzed on the survival of PC3-R cells.

Results: GW4869 (10-20 μM) treatment of PTX resistant PCa cells significantly reduced the release of small EVs (50-100 nm size range) while increasing the release of larger EVs (> 150 nm in size), and inhibited their clonogenicity. Moreover, GW4869 (5-20 μM) treatment (24-72h) significantly inhibited the survival of PC3-R cells in a dose-dependent manner. We observed a similar growth inhibition with both imipramine (5-20 μg/mL) and DMA (5-20 μg/mL) treatment in PC3-R cells. Furthermore, GW4869 treatment (IP) in mice bearing PC3-R

xenografts significantly reduced the tumor weight (65% reduction, $P = 0.017$) compared to the vehicle-treated control mice without causing any noticeable toxicity.

Conclusion: Inhibiting the release of EVs could sensitize the resistant PCa cells to chemotherapy.

Keywords: Prostate cancer, extracellular vesicles, GW4869, chemoresistance, paclitaxel

INTRODUCTION

Prostate cancer (PCa) is one of the most predominant types of cancer in men, accounts for 26% of all cancer cases, and remains one of the leading causes of death[1]. Most of the PCa cases show an indolent stage with no or less risk of mortality; however, many patients develop intermediate or high-risk locally advanced or metastatic phenotype. It is well established that the growth and progression of the PCa are dependent on androgen; thus, androgen deprivation therapy (ADT) is the most accepted practice as the first-line treatment for metastatic /disseminated PCa in the clinical setting[2,3]. Despite the initial response to the ADT, most patients develop castration-resistant PCa (CRPC), characterized by increased serum levels of prostate-specific antigen (PSA) and increased growth and metastasis of primary tumor, leading to disease aggressiveness despite castrate serum levels of testosterone[4,5]. Treatment of patients with CRPC is one of the major challenges for the development of effective treatment therapy for PCa. Many clinical trials have demonstrated the effectiveness of taxol (paclitaxel and its semisynthetic analog docetaxel) for the treatment of CRPC patients due to its effectiveness in prolonging the overall survival[6-8]. Docetaxel has already been approved by the United States FDA for the treatment of CRPC patients.

Paclitaxel (PTX) was the first member of the taxane family approved for cancer chemotherapy. PTX is used in the treatment of CRPC (in combination with ADT) and possesses anti-neoplastic properties by promoting and stabilizing the polymerization and assembly of tubulin required for microtubule formation during the cell cycle, which ultimately causes cell cycle arrest leading to apoptosis[9]. After multiple treatment schedules with PTX, cancer cells develop resistance that leads to a poor prognosis. The development of drug resistance by cancer cells is one of the major challenges in cancer therapy. However, the mechanism of drug resistance is largely unknown but often mediated by insufficient availability of the drug to the target cell. Many mechanisms which are adopted by cancer cells to develop drug resistance have been demonstrated, including drug efflux by membrane-bound efflux proteins, increased interstitial fluid pressure that impairs drug uptake, acidic extracellular microenvironment and hypoxia, hyperglycemia, cellular rewiring, altered drug metabolism, and mutation in drug's target[10-16]. PTX resistant DU145 and PC3 cells have been shown to overexpress multiple drug resistance gene (MDR-1)-encoded P-glycoprotein and enhanced expression of F-actin polymerization[17] as a mechanism to develop resistance against PTX. The direct expulsion of the anticancer drug through extracellular vesicles (EVs) may contribute crucially to the development of chemoresistance. In an earlier study, Shedden *et al.* demonstrated the efflux of anticancer drug doxorubicin through EVs, hence preventing the drug from reaching its target site and also decreasing the available drug concentration for its anti-neoplastic effect[18]. Moreover, it has also been reported that the EVs isolated from drug resistant-cancer cells can efficiently transfer proteins involved in drug efflux pumps to the drug-sensitive cells resulting in the acquisition of MDR[19,20]. Several studies have shown the involvement of EVs released from drug-resistant cells in transmitting resistance to the other cells (reviewed in[21]).

EVs are membranous vesicles released by all cell types and can contain nucleic acids, lipids, and proteins as cargos, which are representative of the secretory cell and its biological behavior[22-24]. EVs are classified into exosomes (~30-150 nm), ectosomes/microvesicles (100-1000 nm), apoptotic bodies (1-5 μM), and large

oncosomes (1-10 μM) on the basis of their size and released pathway[22,25]. The role of EVs in cell-to-cell communication in modifying tumor microenvironment, preparation of pre-metastatic niche, and helping the formation of metastatic foci at distant sites in cancer is well established. Many studies have also suggested the role of EVs in mediating drug resistance in cancer[23,26]. Inhibition of the EVs biogenesis or release by cancer cells may be helpful in preventing the metastasis and the spread of drug resistance to the sensitive cells.

Inhibition of EV biogenesis using specific inhibitors can be used to evaluate the involvement of EVs in cancer cell proliferation and metastasis. GW4869, dimethyl amiloride (DMA), and imipramine are known chemical compounds commonly used as inhibitors of the EV biogenesis/secretion[27]. GW4869 acts as a non-competitive inhibitor of membrane neutral sphingomyelinase (nSMase) enzyme, which is responsible for the generation of lipid ceramide through the hydrolysis of the membrane lipid sphingomyelin and inhibits the release of small EVs/exosomes[28,29]. Imipramine is a tricyclic antidepressant that also exhibits an inhibitory activity on acid SMase and inhibits the generation of ceramide[27]. DMA inhibits H^+/Na^+ and Na^+/Ca^{2+} exchangers and prevents the calcium gradient establishment which is required for the release of exosomes from the cells[30,31]. Earlier, we have reported that GW4869 and DMA treatment inhibits the survival and clonogenicity of PCa cells under both normoxic and hypoxic conditions, as well as the survival of enzalutamide-resistant PCa cells[31,32]. Earlier studies have also suggested that GW4869 reduces the chemoresistance in the cancer cells by inhibiting the EV release[33,34]. Richards *et al.* reported that treatment of gemcitabine to cancer-associated fibroblasts causes increased exosome secretion, which ultimately increases the rate of proliferation in chemosensitive pancreatic epithelial cancer cells and their survival, subsequent treatment with GW4869 significantly reduced their survival[34]. In the present study, we reported the inhibitory effects of GW4869 on the survival of PTX-resistant PCa cells, both *in vitro* and *in vivo*. We also found that the concentration and size distribution of EV released by PTX-resistant PCa cells was significantly affected by the GW4869 treatment.

MATERIALS AND METHODS

Cell lines and reagents

In this study, we used the previously developed PTX resistant human PCa PC3 and DU145 cells[35,36]. PTX resistant PC3 (PC3-R) and DU145 (DU145-R) cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 100 units/mL final concentration of penicillin-streptomycin (Gibco Laboratories, Gaithersburg, MD), and 0.2 μM final concentration of PTX (in DMSO). Cells were incubated at 37 °C in CO_2 incubator at a 5% CO_2 concentration (Heracell VIOS 160i; ThermoFisher, Waltham, MA). GW4869 was purchased from Sigma-Aldrich (St. Louis, MO) and 5-(N,N-Dimethyl) amiloride hydrochloride (DMA) from ThermoFisher (Waltham, MA), while imipramine was purchased from Selleckchem (Pittsburgh, PA).

EVs isolation

Total EVs were isolated from both PC3-R and DU145-R cells after treatment with 10 and 20 μM doses of GW4869. Cell number was counted, and condition media was collected after 24 and 48 h following treatment with GW4869. Next, EVs were isolated from conditioned media using ExoQuick™ (System Biosciences, Palo Alto, CA) precipitation method as described earlier[37]. Briefly, the cell culture conditioned media was first centrifuged at 500 *g* for 5 min, 2,000 *g* for 10 min, 10,000 *g* for 30 min at 4 °C to remove large-sized vesicles. Finally, EVs were isolated using ExoQuick following the manufacturer's recommendations. EV pellet was dissolved in filtered Dulbecco's phosphate-buffered saline (DPBS).

Nanoparticle tracking analyses

The concentration and size distribution of the EVs were analyzed using Nanosight NS300 (Malvern Instruments, UK) as described earlier[38].

MTT assay

PC3-R cells (~1,000) were seeded in 96-well plates and, after 24 h, treated with different doses of GW4869 (5, 10, and 20 μM), DMA (5, 10, and 20 μg/mL), and imipramine (5, 10, and 20 μg/mL). The MTT assay was performed 24, 48, and 72 h after treatment. Briefly, 20 μL MTT (5 mg/mL in PBS) was added to each well and incubated in the dark for 2 h, and then 200 μL of DMSO was added to dissolve the formazan crystals. Finally, absorbance was measured at 560 and 650 nm.

Clonogenic assay

PC3-R and DU145-R cells (200 cells per well) were seeded in 6-well plates and treated with 10 and 20 μM doses of GW4869. After 6-7 days of treatment, colonies (≥ 50 cells) were counted under a microscope. Thereafter, cells were fixed in methanol, stained with crystal violet solution, dried, and photographs were captured.

Tumor Xenograft

All mice were housed and all experiments performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest University Health Sciences (Winston-Salem, NC). Male athymic nude mice (*nu/nu*) were purchased from Envigo (Indianapolis, IN) at 4-6 weeks of age and were given ad libitum food and water on a 12-h light-dark cycle.

PC3-R cells were collected on the day of injection and resuspended in serum-free media. An equal volume of Matrigel matrix (Corning, Bedford, MA) was added to the cells and kept on ice. Mice were anesthetized with Isoflurane at 2%-4%. Using a 1cc syringe with a 27g needle (BD, Franklin Lakes, NJ), ~2.1×10^6 cells were injected subcutaneously on each flank of all the mice. As soon as the tumors were visible, they were measured twice a week with calipers, and volume was calculated as Width $(mm)^2$ × Length (mm) × 0.52 = Volume $(mm)^3$. At the time when the tumors were approximately 50-100 mm^3, mice were divided into two groups. Mice were treated with either GW4869 (Sigma, St. Louis, MO) or vehicle [Vehicle control (VC)], 5% DMSO in 0.9% Sodium Chloride (Baxter, Deerfield, IL). Mice were injected intraperitoneal (IP) with 2.5 mg/kg GW4869 or vehicle (200uL) 6 days a week for 21 days, and then the dose of GW4869 was increased to 5.0 mg/kg. Throughout the experiment, the body weight of each animal was regularly measured. Any mouse with xenograft volume approaching the size limit or other parameters (e.g., necrosis) defined by IACUC was sacrificed, and tissues were collected. At the end of the experiment, animals were sacrificed using CO2 asphyxiation, and blood and tumors were harvested. Plasma was isolated from the blood and stored at -80 °C until further use.

Tissue processing and immunohistochemistry

Tumor tissues were processed, and 5 μm formalin-fixed and paraffin-embedded (FFPE) sections were analyzed for CD63 expression by processing and immunohistochemistry (IHC) as described by us previously[39]. CD63 primary antibody was purchased from ThermoFisher (Waltham, MA) and detection system (ImmPRESS-AP horse anti-rabbit IgG polymer detection kit) with secondary antibody from Vector laboratories (Burlingame, CA). All the immunostained slides were scanned by NanoZoomer (Hamamatsu, Japan) at 20×. The CD63 immunostaining scoring was conducted manually by evaluating the intensity of staining and percentage of stained cells: intensity was given scores of 0-3 (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = intense staining), and the percentage of immunopositive cells was given scores 0%-100%. The 2 scores were multiplied to obtain the final IHC score (between 0-300).

EVs isolation from plasma

Isolation of total EVs from mice plasma was performed as described by us previously[38,40]. Briefly, plasma was diluted in PBS and centrifuged at 500 *g* for 5 min, 2,000 *g* for 10 min, followed by 10,000 *g* for 30 min at 4 ℃ to remove the larger sized vesicles. Finally, EVs were isolated using ExoQuick following the manufacturer's recommendations (System Biosciences, Palo Alto, CA). EV pellet was dissolved in filtered DPBS.

Western blotting

Briefly, 35 µL of EVs isolated from mice plasma were denatured directly in 5× loading buffer and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% Tris-glycine gel. The separated proteins were transferred onto nitrocellulose membrane, and after blocking with 5% non-fat milk powder (w/v) in Tris-buffered saline with tween (TBS-T, 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20), the fresh membrane or stripped membrane was probed with anti-syntenin (Abcam, Waltham, MA) or anti-CD63 (Invitrogen, Waltham, MA) or anti-GOLGA2 (Novus Biologicals, Littleton, CO) primary antibodies for overnight incubation at 4 ℃. Further, in each case, the membrane was washed 3 times with TBS-T and incubated with appropriate secondary antibodies before visualization by the ECL detection system. The autoradiograms/ bands were scanned and quantified using Image J (version 1.53e) using corresponding bands in Ponceau stained membrane as the loading control.

Statistical analysis

Student's *t* test (unpaired) was used to examine the statistical significance ($P < 0.05$) of differences between control and treatment groups.

RESULTS

Effect of GW4869 treatment on the EVs secretion by PC3-R cells

PC3-R cells were treated with 2 different doses (10 µM and 20 µM) of GW4869, and conditioned media was collected after 24 and 48 h. A significant shift in the size distribution of EVs was observed at both 24 and 48 h, with a more noticeable difference at 10 µM dose [Figure 1A]. Though no difference in total particle number secreted per million cells was observed at 24 h, a significant increase in the particle number per million cells was observed at the higher dose of GW4869 with 48h of treatment [Figure 1B, lower panel]. We also noticed a significant increase in the mean size of EVs isolation at 24 and 48 h with both the doses, though the increase was more prominent with the 10 µM dose [Figure 1C]. Analysis of change in EVs in different size ranges showed that the proportion of EVs in size range 50-100 nm was significantly decreased, while the proportion of > 150 nm sized EVs increased significantly with 10 µM dose of GW4869 at both 24 h and 48 h [Figure 1D]. Treatment of PC3-R cells with a 20 µM dose of GW4869 resulted in a 35% reduction (though statistically non-significant) in the proportion of 50-100 nm sized EVs and increased the proportion of 150-200 nm and 200-250 nm sized EVs at 24 h time [Figure 1D, upper panel]. Furthermore, GW4869 at 20 µM dose marginally (statistically not significant) reduced the proportion of 50-100 nm sized EVs and increased 150-200 nm sized EVs at 48 h [Figure 1D, lower panel].

GW4869 reduces the survival and clonogenicity of PC3-R cells

Next, an MTT assay was performed to assess the viability of the PC3-R cells after GW4869 (5-20 µM) treatment for 24-72 h. A significant reduction in cell viability was observed compared to control in treatment with a 20 µM dose at all the time points. We observed a decrease in cell viability with a 10 µM dose at either early (24 h) or late time (72 h); however, with a 5 µM dose, we only observed a statistically significant decrease at 72 h [Figure 2A].

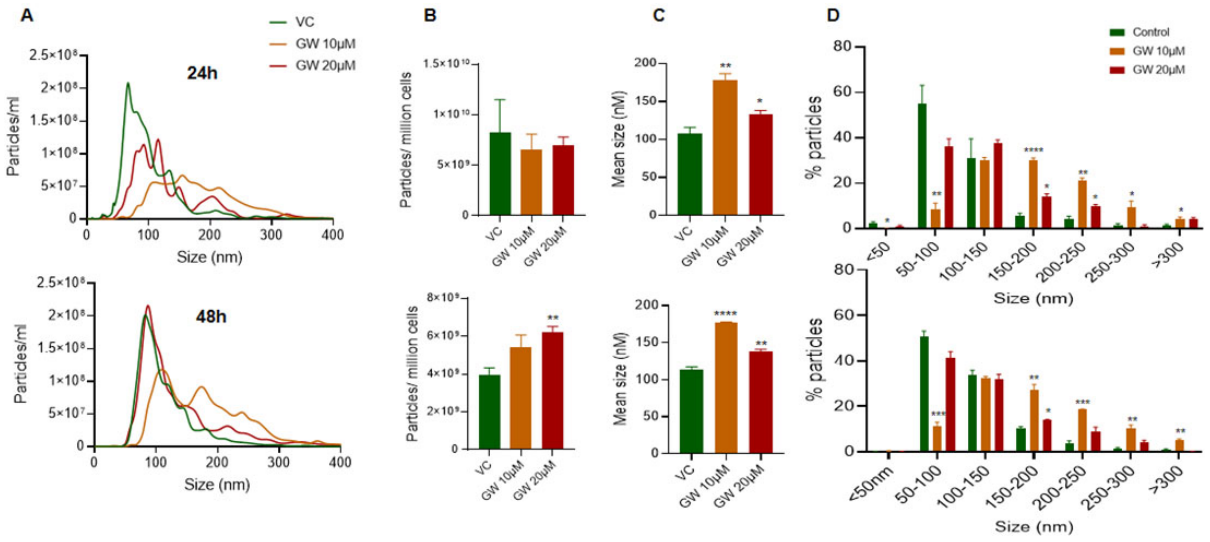

Figure 1. Characterization of EV concentration and size distribution following GW4869 treatment in PC3-R cells. EVs were isolated from the conditioned media of paclitaxel-resistant PC3-R cells following 24 h (upper panel) and 48 h (lower panel) of treatment with GW4869 (10-20 μM) and characterized for concentration and size distribution by NTA. (A) Concentration and size distribution of EVs isolated from DMSO (VC) or GW4869 (10 and 20 μM) treated cells are represented with green, orange, and red colors, respectively (n = 3). Each line represents the mean of three samples, and an average data of 5 videos of 30 sec each was used for each sample. (B-C) Total EV concentration per million cells and mean size are plotted. (D) Size distribution of EVs is presented as percent particles in the mentioned size range. Each bar represents mean ± SEM (n = 3). VC: Vehicle control; *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.0001.

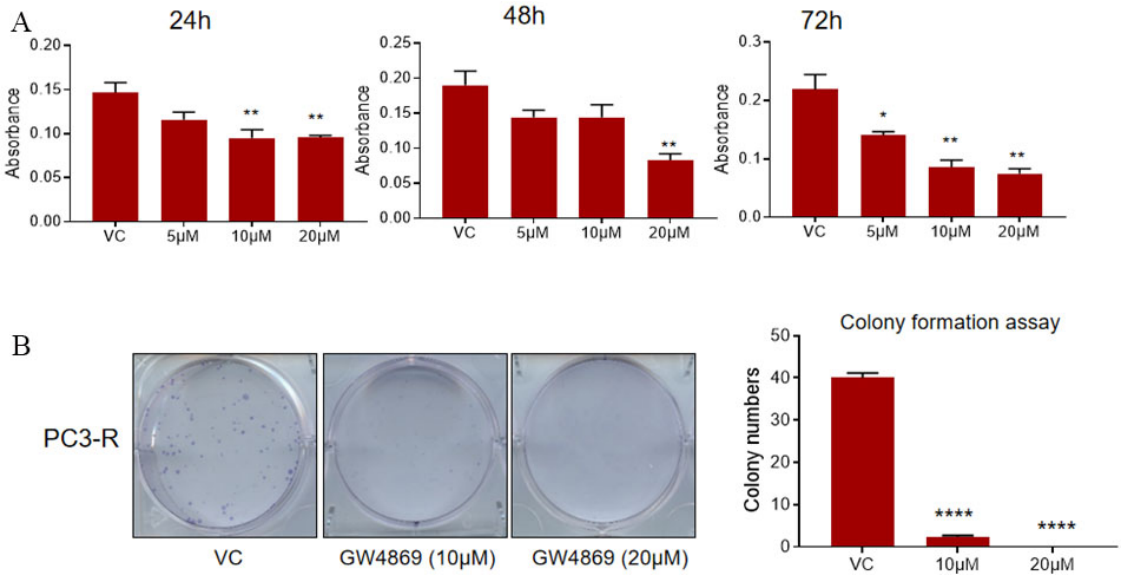

Figure 2. Effect of GW4869 treatment on the survival and clonogenicity of PC-R cells. (A) Paclitaxel-resistant PC3-R cells were treated with DMSO (VC) or GW4869 (5-20 μM), and cell viability was measured in MTT assay after 24, 48, and 72 h. Data are presented as mean ± SEM (n = 5 replicates per group). *P < 0.05, **P < 0.005. (B) Colony formation was measured in PC3-R cells after GW4869 treatment (10 and 20 μM) as described in the methods. Representative images are shown (left panel), and the colony number is presented as mean ± SEM (n = 3 replicates per group). ****P < 0.0001. VC: Vehicle control.

Since PC3-R cells showed a prominent effect of GW4869 treatment mostly at later time points, a colony formation assay was performed to assess the effect on the clonogenicity of PC3-R cells following GW4869

treatment. In colony formation assay, a significant reduction in the number of colonies was observed in PC3-R cells at both 10 and 20 μM doses [Figure 2B].

GW4869 administration inhibits the PC3-R xenograft tumor growth in nude mice

The anti-tumorigenic potential of the GW4869 was evaluated in the nude mice by implanting PC3-R cells subcutaneously in two groups (control and GW4869). No significant change in the body weight of control and GW4869 administered mice was observed after 40 days [Figure 3A]. The tumor volume from the mice that survived at each time point was measured, and the animals that survived by the end of the study were sacrificed on the 44th day after PC3-R cells implantation. A statistically significant difference in tumor volume was observed compared to the control group towards the end of the experiment [Figure 3B]. The tumor tissue weight was also reduced significantly (65% reduction, $P = 0.017$) with GW4869 treatment compared to the control group [Figure 3C].

The expression of CD63 (typical small EVs/exosome marker) in tumor tissues was analyzed by IHC to identify the effect of GW4869 on EVs biogenesis in the tumor tissue. However, no significant difference in the CD63 expression was observed between control and GW4869 treated mice [Figure 3D]. Further, total EVs were isolated from the plasma of control and GW4869 treated animals and analyzed by NTA. No significant difference in the concentration and size distribution was observed between the control and GW4869 groups [Figure 3E]. However, Western blot analysis showed a 95% decrease in the expression of syntenin and about a 70% decrease in the expression of CD63 [Figure 3F and G]. GOLGA2 (Golgin A2) was used as a potential negative marker for EV cargo.

DMA & imipramine treatment reduce the survival of PC3-R cell

To confirm that the observed effect of GW4869 on PC3-R cells and tumor growth is mediated by inhibition of EVs secretion, we used two other compounds (DMA and imipramine), known to inhibit EV biogenesis [reviewed in[27]]. PC3-R cells were treated with different concentrations of DMA and Imipramine to evaluate their effect on cell viability by MTT assay. Treatment with different concentrations (5-20 μg/mL) of DMA showed a significant reduction in the viability of PC3-R cells at all time intervals [Figure 4A]. Similarly, imipramine treatment (5-20 μg/mL) also reduced the PC3-R cell's survival at all-time intervals with all concentrations [Figure 4B].

GW4869 treatment inhibits EVs secretion and clonogenicity of DU145-R cells

The effect of GW4869 treatment was also assessed on EVs secretion by PTX resistant DU145-R cells. GW4869 treatment did not significantly affect the particles per million cells at both 24 h and 48 h [Figure 5A and B]. We observed a significant increase in the mean size of the particles with a 10 μM dose at both 24 and 48 h, while no change was observed with a 20 μM dose [Figure 5A and B]. Consistent with PC3-R cells, GW4869 treatment reduced the proportion of 50-100 nm sized EVs by more than 50% (though statistically not significant) at 10 μM dose and increased the proportion of > 150 nm sized EVs at 24 h [Figure 5A]. The treatment with a 20 μM dose of GW4869 did not show any significant effect on EVs size at 24 h [Figure 5A]. We also observed a significant reduction in the proportion of 50-100 nm sized EVs with 10 μM dose of GW4869 and a statistically significant increase in 150-200 nm, 200-250 nm, and 250-300 nm sized EVs at 48 h. Further, a higher dose of GW4869 (20 μM) increased the proportion of 150-200 nm and 250-300 nm sized EVs after 48 h of treatment without significantly affecting the proportion of 50-100 nm sized EVs [Figure 5B].

The colony formation assay showed that GW4869 treatment (10 and 20 μM) resulted in a significant reduction in the number of colonies formed by DU145-R cells [Figure 5C].

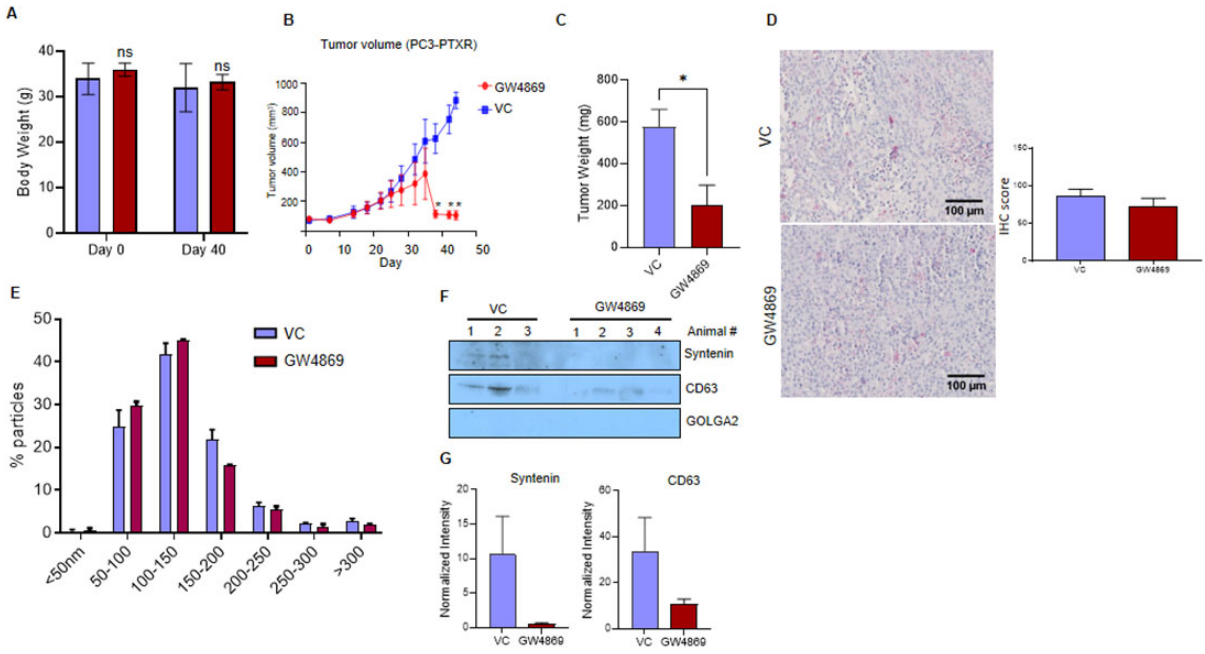

Figure 3. *In vivo* effect of GW4869 treatment on the growth of paclitaxel-resistant PC3-R cells' xenografts. Male athymic nude mice were treated with vehicle or GW4869, and various study parameters were assessed as described in the methods. (A) Average body weight (mean ± SEM) at the start and day 40 of the study for control mice (n = 3) and GW4869 treated mice (n = 4) mice. (B) Average xenograft volume (mean ± SEM) in control and GW4869 treated mice survived at measured time points is presented. (C) In the end, mice were sacrificed, xenografts were excised, and their weight was measured. Average tumor weight in the control group (n = 6 xenografts) and the GW4869 group (n = 8 xenografts) is presented as mean ± SEM. Unpaired t test was used to calculate the statistical significance, *P = 0.017. (D) CD63 expression in xenograft tissues from the vehicle control (VC) group and GW4869 group (n = 6 each) was measured by IHC. For each image, ten random areas were analyzed for IHC scoring as described in the methods. Mean IHC scores are presented as mean ± SEM in the bar diagram. Representative images are shown. (E) EVs isolated from the plasma of control (n = 3) and GW4869 treated mice (n = 3) were analyzed by NTA, and the size distribution (mean ± SEM) of EVs was plotted as a percentage of total EVs. (F) EVs from VC (n = 3) and GW4869 treated (n = 4) mice were used for the analysis of syntenin, CD63, and GOLGA2 by Western blotting. Representative immunoblots are shown. (G) Densitometry analysis of syntenin and CD63 expression was performed and normalized with corresponding band intensity in Ponceau-stained membrane. The relative band intensity is presented as mean ± SEM.

DISCUSSION

Chemotherapy is the major treatment option for CRPC; however, the acquisition of chemoresistance by PCa cells is considered as the main obstacle in the development of an effective anticancer strategy. Many molecular mechanisms that contribute to the development of chemoresistance in cancer cells have been suggested, including transporter pumps, altered metabolism, alteration in gene expression, epithelial to mesenchymal transition, cancer stemness, hypoxia, and acidic tumor microenvironment[41-43]. Drug-resistant cancer cells utilize EVs to transfer the transporter pumps and other biomolecules involved in pro-survival and anti-apoptotic pathways to the sensitive cells for propagation of chemoresistance[23,44]. Additionally, cancer cells can use EVs for direct loading of drugs and their efflux, which can undoubtedly contribute crucially to the development of chemoresistance in cancer cells. Using different cell line models, Shedden et al. have shown the physical incorporation of doxorubicin in EVs and its expulsion into the media[18]. It indicated that cancer cells secrete the EVs as an intrinsic mechanism to enable the resistant cells to survive under stressful or toxic environments. Further, it is well accepted that in physiological and biochemical stress conditions like hypoxia or an acidic environment, cancer cells secret higher amounts of EVs[31,45,46] as a protective mechanism to remove metabolic and toxic waste. Treatment of PTX resistant PC3-R and DU145-R cells with GW4869 (a selective neutral sphingomyelin phosphodiesterase inhibitor) showed a specific reduction on 50-100 nm sized EVs, which are in the size range of exosomes/ small EVs, though increased

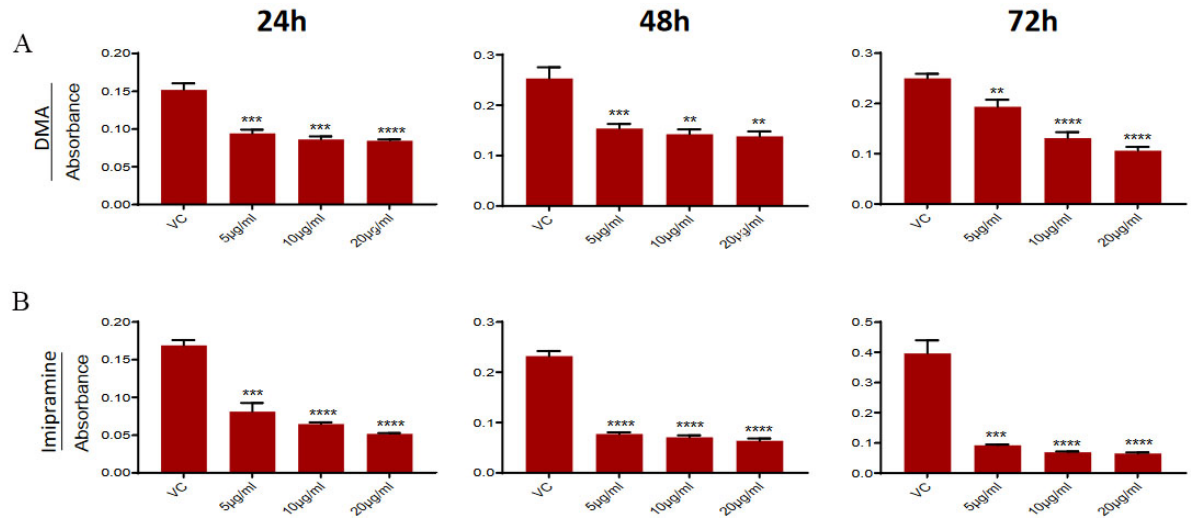

Figure 4. Effect of DMA and imipramine treatment on the survival of paclitaxel-resistant PC3-R cells. PC3-R cells were treated with (A) DMA (5-20 μg/mL) or (B) imipramine (5-20 μg/mL) for 24-72 h and analyzed for cell viability in the MTT assay. Data are presented as mean ± SEM (n = 5 replicates per group). $^{**}P < 0.005$, $^{***}P < 0.0005$; $^{****}P < 0.0001$.

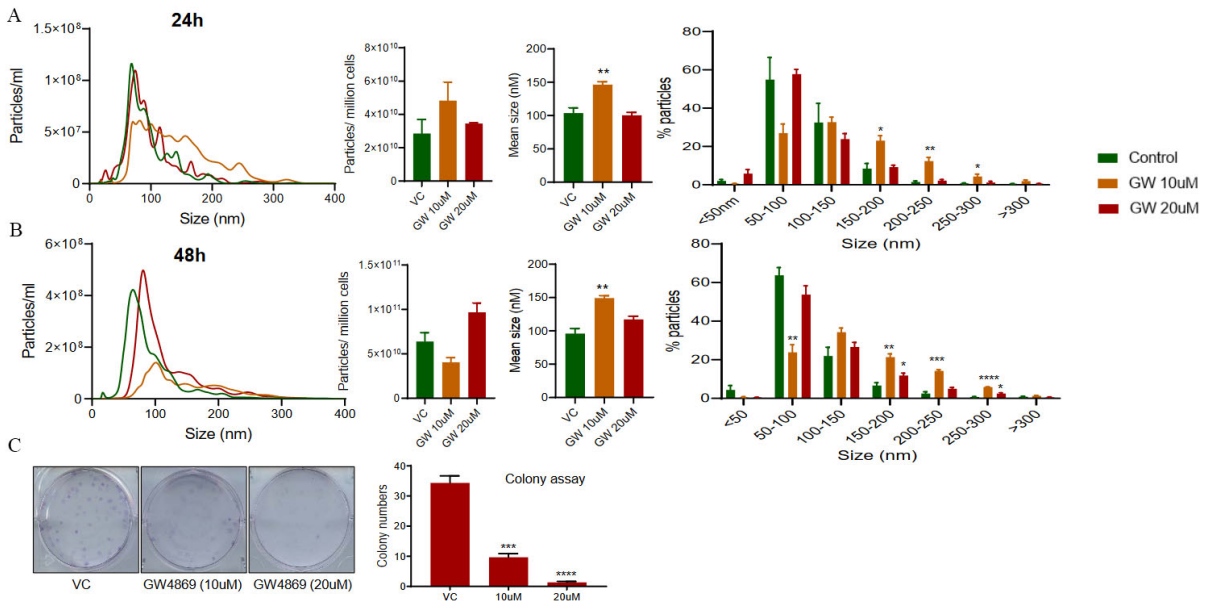

Figure 5. Effect of GW4869 treatment on paclitaxel-resistant DU145-R cells. EVs were isolated from the conditioned media of paclitaxel-resistant DU145-R cells following 24 h (upper panel) and 48 h (lower panel) of treatment with GW4869 (10-20 μM) and characterized for concentration and size distribution by NTA. (A-B) Each colored line in the left panel represents the mean of three samples, and an average data of 5 videos of 30 s each was used for each sample. Concentration (particles/mL)-size distribution, average EV concentration per million cells, average size, and percent particles for various size ranges are presented. Each bar represents mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.005$ $^{***}P < 0.0005$ $^{****}P < 0.0001$. (C) Colony formation was measured in DU145-R cells after GW4869 treatment (10 and 20 μM) as described in the methods. Representative images are shown (left panel), and the colony number is presented as mean ± SEM (n = 3 replicates per group). $^{***}P < 0.0005$, $^{****}P < 0.0001$.

the proportion of larger size (> 150 nm) EVs. The observation was in line with the previous report suggesting the treatment of human breast cancer cell line SKBR-3 with 5 μM GW4869 or siRNA against sphingomyelin phosphodiesterase 2/3 (also known as nSMase) resulted in a significant reduction of < 100 nm sized vesicles and increased quantities of vesicles with a size range of 100-200 nm; while overexpression

of sphingomyelin phosphodiesterase-3 (also known as nSMase2) decreased the amount of larger-sized (100-200 nm) vesicles[47]. Interestingly, the increase in the EV fraction, following GW4869 treatment for 16 h, collected after 14,000 × *g* centrifugation (larger sized vesicles) was suggested to be stemming from plasma membrane representing MVs. Moreover, higher content of sphingomyelin in the MVs membrane was reported with respect to the overall cell membrane lipid composition, while no difference in EVs collected at 100,000 × *g* fraction (small EVs) was observed. Therefore, inhibiting the nSMase with GW4869 can interfere with the lipid composition, which may inhibit small EVs/exosome secretion but increase the secretion of larger EVs/MVs[47]. Interestingly, in line with our observation, the treatment of SKBR3 cells with GW4869 was shown to affect the loading of proteins (including syntenin) in EVs and carry less protein per EV[47].

Several studies have indicated that EVs or exosome secretion can be inhibited by the use of GW4869, imipramine, and DMA[28,31,32,48-52]. Interestingly, treatment of PTX-resistant PCa cells with these known exosome biogenesis inhibitors resulted in a significant decrease in cell viability, especially in a long-term clonogenic assay. Similar inhibition of EVs secretion with GW4869 treatment leading to decreased growth of B16BL6 cells was reported earlier[53]. We have also previously reported that the treatment with GW4869 and DMA decreased the cell survival of the enzalutamide-resistant PCa cells[32] as well as clonogenicity of PCa cells under both normoxic and hypoxic conditions[31].

The study by Corcoran *et al.* has shown that docetaxel resistant variants of DU145 and 22Rv1 cells transfer the docetaxel resistance to sensitive cells (DU145, 22Rv1 and LNCaP) partly through MDR-1/P-gp transfer, suggesting the role of exosomes in the propagation of chemoresistance[54]. Moreover, other studies have reported the inhibition of EVs secretion in cell culture and/or suppression of tumor growth in mice after GW4869 treatment[53,55-57]. *In vivo* experiment in the present study indicated that GW4869 treatment decreases the tumor growth significantly without affecting the overall bodyweight of the mice. Although, we did not observe a significant change in the total EV number isolated from the plasma of these mice and CD63 expression in the xenograft tissues. However, we observed a strong decrease in the loading of known exosomal cargo proteins (syntenin and CD63). Earlier, Dinkins et al. have shown the reduction in protein concentration and reduced levels of Alix (a marker for exosomes/EVs) in EVs isolated from the mice serum treated with intraperitoneal injections of 100 µg GW4869 (~4 µg/g) daily for 5 days[51]. The effect of GW4869 treatment on the concentration of EVs was not measured in this study[51]. Similarly, intraperitoneal treatment of C57BL/6J mice with GW4869 (1.25 mg/kg) decreased airway-secreted EVs *in vivo*, as measured directly with NTA and indirectly with miRNAs expression[58]. Essandoh *et al.* also showed that treatment of wild-type mice with GW4869 at a dose of 2.5 µg/g of body weight reduced the levels of serum exosomes by 33%. Though, the concentration of exosomes was measured indirectly by acetylcholinesterase (AChE) activity assay[59]. Interestingly, in an established multiple myeloma (MM) mouse model, intraperitoneal injection of 1.25 µg/g GW4869 for 2 weeks suppressed MM tumor growth, though no association with nSMase expression and sensitivity to GW4869 was observed. Moreover, the molar levels of sphingomyelin decreased while ceramide increased in GW4869 treated MM cells for a specific duration, suggesting that the cytotoxic effect of GW4869 may not be through direct nSMase2 inhibition[60]. Overall, it appears that the dose and treatment duration of GW4869, as well as the cancer-type or study model, could also impact its effect on EV biogenesis and secretion in biofluids.

The mechanism by which GW4869 inhibits the survival and growth of the PTX-resistant PCa cells reported in this study could be partly through the inhibition of smaller EVs secretion (50-100 nm). The GW4869 mediated inhibition of the growth of the PTX resistant cells *in vitro* and *in vivo* indicated its usefulness in combating the chemoresistance in the PCa. The potential of the GW4869 as a combinational therapy to the PCa along with chemotherapeutic drugs needs to be explored further for the better management of chemoresistant PCa.

DECLARATIONS

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Authors' contributions

Conceived the study: Deep G
Designed the experiments: Deep G, Kumar A
Performed the experiment: Kumar A, Kumar P, Sharma M, Kim S, Singh S
Analyzed the data: Deep G, Kumar A
Wrote the manuscript: Deep G, Kumar A, Kumar P, Kridel SJ
Proof read the manuscript: Kumar A, Kumar P, Sharma M, Kim S, Singh S, Kridel SJ, Deep G

Availability of data and materials

Raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All experiments performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Wake Forest University Health Sciences (Winston-Salem, NC).

Consent for publication

Not available.

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Cancer Drug Resistance

Perspective

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AdipoRon and Pancreatic Ductal Adenocarcinoma: a future perspective in overcoming chemotherapy-induced resistance?

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Abstract

The latest scientific knowledge has provided additional insights accountable for the worst prognosis for pancreatic ductal adenocarcinoma (PDAC). Among the causative factors, the aptitude to develop resistance towards approved medications denotes the master key for understanding the lack of improvement in PDAC survival over the years. Even though several compounds have achieved encouraging results at preclinical stage, no new adjuvant agents have reached the bedside of PDAC patients lately. The adiponectin receptor agonist AdipoRon is emerging as a promising anticancer drug in different cancer models, particularly in PDAC. Building on the existing findings, we recently reinforced its candidacy in PDAC cells, proposing AdipoRon either as a suitable partner in gemcitabine-based treatment or as an effective drug in resistant cells. Crossing the current state-of-the-art, herein we provide a critical perspective on AdipoRon to figure out whether this receptor agonist can potentially be considered a future therapeutic choice in overcoming chemotherapy-induced resistance, expressly in PDAC.

Keywords: PDAC, AdipoRon, gemcitabine, resistance

INTRODUCTION

Considered as one of the most aggressive malignancies, pancreatic ductal adenocarcinoma (PDAC)

accounts for almost all cases of pancreatic cancer[1]. Due to the lack of specific and sensitive biomarkers, diagnosis usually happens too late, or rather when the advanced metastatic stage has already occurred[2]. The disappointing outcomes provided by the available therapeutic regimes have further contributed to worsening PDAC prognosis, whose five-year survival rate currently stands at 10%[3]. Regrettably, according to the existing predictive studies, PDAC will become the second leading cause of cancer death in the next decade.

Even though a combination of surgery and adjuvant chemotherapy represents the approved curative regimen for PDAC management[4], due to the advanced or metastasized stage at diagnosis, not more than 20% of patients can undergo surgical removal[5]. Therefore, in both resectable and non-resectable PDAC conditions, the only partially viable therapeutic strategy remains chemotherapy. Consistent with clinical practice guidelines, the first-line treatment involves the use of gemcitabine, which can be administered in combination with albumin-bound paclitaxel or FOLFIRINOX (oxaliplatin, irinotecan, leucovorin, and 5-fluorouracil) treatment cycles[6]. Although both combination therapies have moderately improved either overall or median progression-free survival, the highest incidence of mild to severe adverse reactions has strongly restricted their usage in prolonged regimes[7]. Therefore, gemcitabine represents the only alternative to palliative care sometimes. Disappointingly, while PDAC cells are initially extremely responsive to this cytotoxic agent, most patients easily develop resistance within a few weeks, thus compromising the success rate of the treatment[8].

Underlying the fully fledged mechanism of chemotherapy resistance is very intricate in cancer, given that many determining factors may be involved. Essentially, both innate and adaptive mechanisms can concurrently lead to the PDAC-resistant phenotype[9]. Analogously, native and acquired alterations in either nucleoside transporters or metabolic enzymes may prompt PDAC cells to gemcitabine resistance[10]. Due to its hydrophilicity, cellular uptake is mediated by human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs), although hENT1 accounts for transporting almost the entire amount of gemcitabine into cytosol[11]. Once inside, gemcitabine is first phosphorylated by deoxycytidine kinase (dCK), and then converted to nucleoside diphosphate (dFdCDP) and triphosphate (dFdCTP), successively[12]. Its metabolic inactivation, instead, is regulated by cytidine deaminase (CDA) or, after phosphorylation, deoxycytidylate deaminase (dCTD)[13]. Conversely, gemcitabine can inhibit dCDT activity both directly and through its active metabolite, dFdCTP[14,15]. Considering the gemcitabine-related metabolism as a whole, it is quite clear that changes in hENT1 levels, as well as CDA dysregulations, can play a decisive role in defining the degree of resistance against this chemotherapy drug. In PDAC, for instance, variations in CDA expression or activity have been correlated with impaired gemcitabine responsiveness[16-18]. Although gemcitabine is not considered a canonical target of multidrug resistance-associated proteins (MRPs)[19,20], different studies have displayed a sort of collateral modulation in ATP-binding cassettes (ABCs), expressly in PDAC cells unresponsive to deoxycytidine analog[13,21-23]. Besides the tumor-related mechanisms, stroma may further contribute to the establishment of a non-permissive cytotoxic microenvironment[24], both producing a barrier for drug delivery and influencing cancer cell behaviors[25,26]. Secreting soluble factors, remodeling extracellular matrix, delivering exosomes, reprogramming the metabolic process, and the epigenetic landscape of tumor cells are some of the stroma-related properties leading to resistance[27,28]. In addition, there is an ever-growing awareness of the crucial role driven by cancer cell metabolism in influencing drug response[9,29]. In accordance with this perspective, targeting precise metabolic pathways, which include, among others, glycolysis and mitochondrial oxidative phosphorylation (OXPHOS)[30-32], has recently been recognized as a promising pharmacological approach to overcome chemoresistance in PDAC.

Starting from the adiponectin (Acrp30) evidence in regulating pancreatic homeostasis and malignancies, herein we expressly review the adiponectin receptor agonist AdipoRon as both a potential anticancer agent and gemcitabine sensitizer in PDAC. Providing our personal point of view on criticism and feelings, we try to figure out whether this receptor agonist can potentially be considered a future therapeutic choice in overcoming chemotherapy-induced resistance, expressly in PDAC.

Adiponectin: an insight on its aptitudes in regulating pancreatic homeostasis

Encoded by the *ApM1* gene (adipose most abundant gene transcript 1), Acrp30 is a 244-amino acid adipokine synthesized by adipose cells and assembled in three distinct homopolymers, which differ in molecular weight and affinity for receptors[33]. As one of the most abundant serum-related adipokines, Acrp30 physiologically influences a wide range of cellular functions, including either related or unrelated metabolic pathways[34]. Acrp30 primarily regulates glucose and fatty acids homeostasis, and supplementary beneficial properties have extensively been reported. Acrp30 can indeed mitigate pathophysiological conditions such as inflammation, atherosclerosis, and immune-mediated response[35].

The pivotal role of pancreas in regulating glucose uptake makes this organ extremely vulnerable to Acrp30 signaling, even though variable and inconsistent results still exist side by side. In this respect, while many studies provide evidence supporting the Acrp30-mediated insulin release in mouse islets, in humans, this stimulation appears to be ineffective against either basal or glucose-induced insulin secretion[36,37]. However, there are certain considerations that could elucidate this controversy. Contrary to other adipokines, pancreas-specific Acrp30 knockout mouse models have not been engineered thus far; therefore, all the provided information merely recognizes systemic effects which could influence every single statement. Moreover, glucose levels and resistance status are two additional key issues which could contribute to Acrp30-related responses. In this connection, scrutinizing the insulin-resistant mouse islets, Winzell and colleagues warned that Acrp30 can display a dual opposite effect in stimulating this specific hypoglycemic agent, namely inhibiting insulin secretion at low glucose concentrations and promoting its release under hyperglycemic status[38].

Quite convincing is the Acrp30-mediated role in protecting the maintenance and survival of functional β-cells. The available scientific research provides precise data about the anti-apoptotic properties of Acrp30 towards lipids, ceramides, and cytokines[37,39]. In light of its cytoprotective effects, Acrp30 has also been proposed as a potential target to treat metabolic syndrome involving β-cell dysfunction. However, the lack of compounds capable of stimulating Acrp30 production or mimicking its action has made a chimera of this therapeutic strategy.

Almost no modulations have been reported for the remaining islets components, as well as for the exocrine portion of the pancreas. Albeit β-cells constitute nearly the totality of pancreatic islets, specific unrelated changes in pancreatic functions have been detected for other adipokines. In this respect, inducing variations in membrane potential and glucagon secretion, leptin-mediated regulation has been observed in both mouse and human α-cells[40,41]. Alterations in lipases release were also obtained in response to leptin administration[42]. Instead, resistin, a putative adipocyte-derived hormone involved in insulin resistance and diabetes, significantly increased the secretion of pancreatic amylase, thus worsening inflammatory response and acute pancreatitis severity[43].

Scrutinizing the scientific production, it remains quite unclear why no Acrp30 results have been achieved with respect to other pancreatic components. Surely, the limited available indirect findings suggest a chance that even these portions may be responsive to Acrp30 fluctuations. In cystic fibrosis (CF), for instance, an

autosomal recessive inflammatory disorder that consistently results in pancreatic exocrine dysfunction[44], Acrp30 serum levels are usually higher compared to healthy subjects[45,46]. Acrp30 modulations have also been reported in non-functional pancreatic cells, particularly in vascular endothelial cells (VECs), where an unusual overexpression in this adipokine may serve as a protective angiogenic factor in mice fed a high-fat diet[47].

Across the controversial relationship between adiponectin and PDAC

As recently examined in a theoretical study, the vast majority of the Acrp30-mediated functions are carried out by two highly homologous seven-transmembrane helices, termed AdipoR1 and AdipoR2, which recognized the canonical membrane receptors for this kind of adipokine[48]. Once recognition and binding have occurred, Acrp30 promotes the recruitment of adaptor protein APPL1, which in turn activates AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor α (PPARα)[35]. Besides regulating energy homeostasis, Acrp30-related intracellular targets are actively engaged in controlling several signaling pathways that, depending on cell specialization, may lead to opposite effects[49]. The third receptor in order of discovery is a calcium-dependent adhesion molecule, also referred to as T-cadherin, whereby a lack of its intracellular domain may serve as Acrp30 co-receptor for unknown signaling[50].

Although the presence of Acrp30 receptors has not been confirmed in all pancreatic-derived cells yet, its involvement in PDAC initiation and progression has extensively been assessed over the years. The largest part of the existing preclinical studies designates Acrp30 as an effective anticancer molecule in PDAC models[51,52]. There is one piece of evidence suggesting a tissue-dependent outcome on tumorigenesis, which proposes an unconventional Acrp30 role in promoting PDAC growth[53]. The conflicting findings obtained by Huang and colleagues can be explained by the specific strain employed for their *in vivo* experiments. Specifically, inoculating H7 and Panc02 cells in Acrp30-KO and Acrp30-WT C57BL/6 mice, they observed that the size and weight of the resulting tumor in Acrp30-WT mice were larger compared to those achieved in Acrp30-KO. However, apart from connecting these results directly with the Acrp30 action, no accounts were taken of the potential metabolic alterations induced by its systemic ablation, which could constitute a hostile environment for tumor growth per se. However, more accurate experiments involving the use of an inducible system are mandatory to figure out the cause of this controversy.

The clinical findings supporting the Acrp30 involvement in pancreatic malignancy as a putative risk and prognostic factor remain inconclusive. While both US and European nested case-control studies revealed an inverse correlation between the pre-diagnostic plasma levels of Acrp30 and the subsequent risk of developing PDAC[54,55], a recent Mendelian randomized analysis totally rejects this association[56]. Two main limitations make the link between Acrp30 and PDAC risk inconclusive so far: (i) the absence of systematic reviews of randomized controlled trials; and (ii) the limited setting of PDAC patients whose Acrp30 levels were monitored before and during PDAC diagnosis. Moreover, rather than an ordinary relationship with blood levels, PDAC risk could be associated with the expression of specific genetic variants of Acrp30[57]. More persuasive appears the hyperadiponectinemia observed in PDAC instead[58-60]. While this connection could be interpreted as a compensatory response to the PDAC-induced cachexia, Acrp30 plasma levels seem unrelated to either BMI or leptin concentration[58,59]. Therefore, an alternative explanation for this unexpected increase could be a sort of adaptive process for hindering tumor growth. This model could also account for the reduced levels of both Acrp30 receptors observed in PDAC cells relative to normal pancreatic tissue[61]. Nevertheless, further designs are absolutely needed to investigate the above, as well as other possible conjectures. Only few and conflicting assumptions have been stated regarding Acrp30 as a prognostic factor in PDAC[62,63].

AdipoRon and PDAC: more lights than shadows

Due to a combination of both intrinsic and extrinsic limitations, no translation has been conceived for Acrp30 in cancer clinical trials. While the controversial findings have not permitted any subsequent steps involving PDAC patients to date, Acrp30 translation floundered even in cancers showing very convincing preclinical results. The heavy molecular mass and the reduced half-life/stability of Acrp30 have substantially hampered any clinical application in malignancy as well as in other pathological conditions. Nevertheless, the recent characterization of a plausible Acrp30 active and binding site has broken new ground in the design of derived compounds, thus making different druggable options available[64].

In regard to the Acrp30-mediated beneficial properties observed in cell disease models, adiponectin receptor agonists certainly denote the most promising class of compounds having potential therapeutic perspectives. Among the other agonists, AdipoRon is emerging as a fascinating anticancer agent in several malignancies, including osteosarcoma, myeloma, breast and ovarian cancer[65,66]. Standing out as the top-scored in activating AMPK and bonding AdipoR1 and AdipoR2 in murine myeloblast C2C12 cells, AdipoRon is currently recognized as the first orally active adiponectin receptor agonist[67].

Independent research groups have provided convincing evidence supporting the antiproliferative role played by AdipoRon in both *in vitro* and *in vivo* PDAC models[61,68-71]. Unsurprisingly, querying the main scientific databases for AdipoRon, PDAC represents the most mentioned among the tumor models examined thus far. A detailed summary of the key findings obtained in PDAC is discussed hereafter.

Messaggio and co-workers were the first to prove that AdipoRon could suppress tumor growth in PDAC models[61]. Specifically, employing both human and GEMM-derived mouse PDAC cells, they observed reduced proliferation and apoptosis induction in response to AdipoRon administration concurrently. Even though AdipoRon treatments decreased signal transducer and activator of transcription 3 (STAT3) phosphorylation, no experiments have been performed to address its impact on the AdipoRon-mediated action. In this respect, a more comprehensive mechanistic design was proposed by Akimoto and colleagues a year later, who drew attention to RIPK1-dependent necroptosis as the main tool for cell death induction in AdipoRon-treated cells[68]. Nevertheless, based on the obtained results, they did not exclude that caspase-independent apoptosis and autophagy could be similarly involved. Mechanistically, a rise in calcium concentration triggered extracellular signal-regulated protein kinase 1/2 (ERK1/2) and calpain-1 activation, which in turn led to mitochondrial dysfunction and caspase-independent apoptosis induction. Activation of AMPK and p38 signaling were recognized as survival pathways in this specific experimental design, since their pharmacological inhibition enhanced the AdipoRon cytotoxic effects. The impairment of mitochondrial activity was further confirmed by Manley *et al.* in their latest study[69]. Performing Seahorse-based assays, they noticed that AdipoRon treatments not only decreased basal and maximal mitochondrial respiration but also attenuated proton leak. As a compensatory response to defective mitochondrial ATP production, PDAC cells increased anaerobic glycolysis by consuming a greater amount of glucose and producing more lactic acid. Finally, based on these results, the authors recognized glycolysis inhibitors as potential targets to enhance AdipoRon effectiveness. Aimed at providing more insight into the AdipoRon-mediated antitumor properties in PDAC, Takenaga and colleagues revealed a concurrent angiogenesis inhibition in MSS31 endothelial cells, which may cause a shortage of nutrients and oxygen supply in tumor cells[71]. Although AMPK, p38, and ERK1/2 were simultaneously activated by AdipoRon in this cell type, the MEK1 inhibitor U0126 was the only one capable of preserving tube formation. The anti-angiogenic features could also explain the reduced effectiveness observed in the AdipoRon-treated orthotopic pancreatic cancer mice fed a high-fat diet. Specifically, given that obese mice usually show high plasma levels of leptin, this anorexigenic adipokine may dynamically compete with AdipoRon in stimulating endothelial cells, thus overriding pro- rather than anti-angiogenic mechanisms.

AdipoRon improves gemcitabine-mediated outcomes in PDAC cells

While different studies convincingly propose AdipoRon as an anticancer agent in PDAC models, no data support a potential cooperating effect in gemcitabine-based therapy. In this respect, we recently provided evidence of increased responsiveness to gemcitabine when PDAC cells were concomitantly stimulated with AdipoRon. Using multiple biological approaches, we demonstrated that AdipoRon plus gemcitabine significantly decreased tumorigenesis in two distinct PDAC cell lines, MIA PaCa-2 and PANC-1.

In detail, adding AdipoRon to gemcitabine further compromised cell growth and colony-forming ability compared with single treatments, thus suggesting a positive correlation between these two compounds. For this purpose, CompuSyn analysis was subsequently performed, demonstrating a potential synergistic effect in either raising cell-growth inhibition or reducing colony formation. Precisely, this assay revealed a combination index (CI) ranging from 0.59-0.63 in MIA PaCa-2 and 0.05-0.22 in PANC-1. We also recorded G0/G1 and S phase accumulation after the individual release of AdipoRon and gemcitabine, respectively. Remarkably, when these two compounds were administrated together, we noticed different but intermediate features in cell cycle distribution. Without statistically significant variations in SubG1 amount compared to the deoxycytidine analog ($P > 0.05$), AdipoRon plus gemcitabine induced G0/G1 accumulation closer to AdipoRon within 24 h and S-phase arrest similar to gemcitabine after 48 h. The observed variations in cell cycle distribution after AdipoRon, gemcitabine, and AdipoRon plus gemcitabine treatment were further corroborated by cyclins and cyclin-dependent kinase inhibitor levels. Considering the relevance of the ERK1/2 signaling in the AdipoRon-mediated action, we investigated its possible involvement in combination with gemcitabine. Without significant changes in total protein amount, we observed a phospho-ERK1/2 upregulation in combined treatment more than AdipoRon alone. Intriguingly, the usage of PD98059 as a potent MEK1/MEK2 inhibitor partially prevented the enhanced outcomes observed in reaction to AdipoRon plus gemcitabine, thus reinforcing the dynamic influence of this pathway in the AdipoRon-mediated sensitization. To further speculate on the usefulness of AdipoRon-based therapy in PDAC, we finally explored the combination impact in gemcitabine-resistant MIA PaCa-2 cells. While gemcitabine was ineffective in decreasing cell proliferation, AdipoRon treatments, either alone or in combination, hindered cell growth with an inhibition rate of 25% and 43%, respectively. We also observed a reduction in colony numbers after both AdipoRon and AdipoRon plus gemcitabine administration. As in gemcitabine-sensitive cells, AdipoRon was able to increase the G0/G1 phase in resistant ones, while the simultaneous presence of gemcitabine further intensified this tendency.

Critical issues and future perspectives for the AdipoRon usage in PDAC treatment

In light of the latest cancer statistics[3], it is explicit that all clinically approved therapeutic strategies for treating PDAC have provided unsatisfactory responses. While the rest of the cancers are moving toward a chronic management, PDAC remains one of the deadliest malignancies worldwide[72]. Even immunotherapy, which has been designed as the "breakthrough" in cancer treatment, has achieved only weak outcomes in PDAC[73]. Consequently, identifying novel pharmacological approaches is categorically demanded to make PDAC more treatable.

AdipoRon has recently been found to be effective in contrasting PDAC cell growth at preclinical stage[61,68,69,71], but, more interestingly, we recently provided evidence of potential cooperating effects between AdipoRon and gemcitabine[70]. Due to the low rate of radiation and surgery eligibility, gemcitabine-based therapy constitutes the widely used approach in treating PDAC[5]. Regrettably, even though gemcitabine is still considered a cornerstone in PDAC therapy, the chances of developing chemoresistance are extremely high for these patients, and thereby combinatory treatments are usually preferred over single-agent administration. Despite the huge efforts made to provide other pharmacological options, only two gemcitabine partners, erlotinib and nab-paclitaxel, have been approved in clinic[74]. However, their success

Table 1. Metabolic pathways involved in gemcitabine resistance and potentially affected by AdipoRon exposure

Metabolic pathway	Molecular target	Drug inhibitor	Reference
OXPHOS	Complex I	Metformin, rotenone, phenformin	[31]
	Complex IV	Arsenic trioxide	[32]
	Complex V	Oligomycin	[78]
Glycolysis	Hexokinase	2-deoxy-D-glucose	[79]
	Pyruvate Dehydrogenase	CPI-613 (devimistat)	[32]
	LDH-A	N-hydroxyindole-based inhibitors	[80,81]

rate is strictly dependent on several factors, including genetic signatures and performance status scale[75,76]. Recognizing AdipoRon as a potential candidate in gemcitabine-based therapy may provide additional hopes for advanced PDAC patients. However, to be fair, the possibility that AdipoRon may reach the bedside of PDAC patients is currently remote. While AdipoRon has already been tested in animal models as a single agent, showing a high degree of tolerability in normal tissues[68], *in vivo* studies have not corroborated its effectiveness in combination with gemcitabine yet. Recently, another adiponectin receptor agonist has concluded phase 1/2a (NCT04201574) and is currently under evaluation in phase 2/3 clinical trials (NCT04899518). As a peptidomimetic agonist, ALY688 has shown no specific adverse reactions as ophthalmic solution in human patients, and now two distinct concentrations (0.4% and 1%) will be evaluated for their efficacy in subjects with dry eye disease. While extremely promising, a positive ALY688 outcome in human trials would not lead to AdipoRon approval in clinic, but it could pose a driving force for its testing in cancer patients. Adiponectin receptor agonists actually include a heterogeneous group of molecules, and thus they must be investigated individually at every stage. In this respect, dissimilarities in biological behaviors have been observed even between AdipoRon and Acrp30. Characterizing the AdipoRon-mediated mechanism of action in PDAC cells, Akimoto and colleagues noticed that, unlike AdipoRon, neither ERK1/2 activation nor cell death induction was detected in response to Acrp30 administration[68]. These findings bring more doubts about the chemical and biological differences that may exist between these two molecules, as well as whether the AdipoRon-mediated effects are really due to the agonistic action towards Acrp30 receptors.

The ability to make gemcitabine-resistant MIA PaCa-2 cells responsive to AdipoRon denotes a fascinating aptitude that could open up new opportunities in overcoming chemotherapy refractoriness. On the basis of the standing knowledge, there is a rational explanation for corroborating the above biological outcome. Recently, mitochondria have been described to facilitate the survival of stem and dormant cells treated with cytotoxic agents in PDAC[31]. Several mitochondrial-related pathways and mechanisms have been associated with chemoresistance, such as apoptosis, autophagy, and metabolic remodeling[77]. In accordance with this latter operation, in both oncogene-ablated and gemcitabine-treated PDAC cells, targeting OXPHOS significantly shrank tumor recurrence[31,32,78]. Since an OXPHOS impairment has also been observed in response to AdipoRon administration[68,69], it is plausible to imagine a link between the damage that occurred at this organelle and the ability to sensitize PDAC resistant cells to gemcitabine. While the aberrant glycolysis usage is considered a metabolic feature of drug resistance in tumor cells[30,79], after AdipoRon stimulation, this compensatory mechanism could be exploited to completely eradicate the minimal residual disease [Table 1][69,80,81]. Besides the metabolic outlook, another potential mechanistic way to corroborate the AdipoRon-mediated gemcitabine sensitization could be represented by the ABC transporters, which have recently been implicated in chemotherapy resistance, expressly in PDAC[21-23]. Despite no proof currently demonstrating an AdipoRon-induced ABCs modulation, limited findings correlate Acrp30 and some members of this class of transporters. In this respect, Acrp30 has been described to increase both mRNA and protein levels of ABCA1 in hepatocellular carcinoma HepG2 cells[82]. A positive

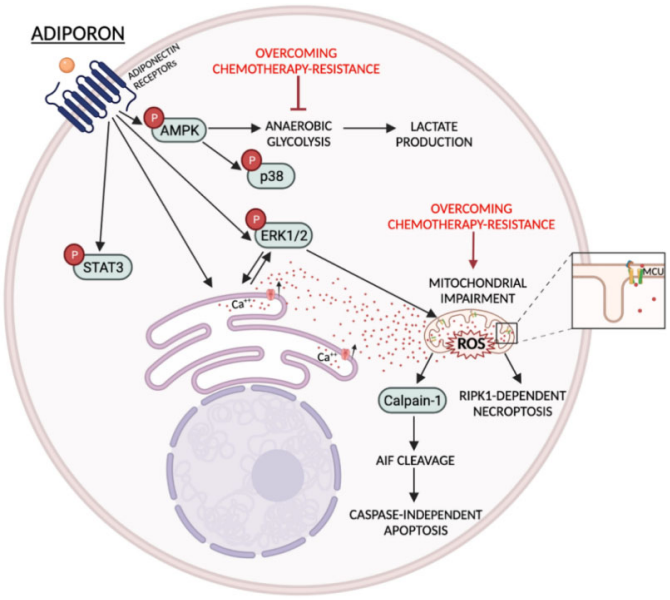

Figure 1. AdipoRon-mediated mechanisms and potential interconnection points to overcome gemcitabine resistance in PDAC. Schematic representation of the main metabolic and signaling pathways regulated by AdipoRon in PDAC cells. Red arrows indicate direct and indirect AdipoRon-related ways to hinder chemotherapy resistance.

correlation between Acrp30 and ABCA1 levels has also been observed in visceral adipose tissue[83]. However, the abovementioned speculation first warrants further investigation about the possible contribution of ABC transporters to gemcitabine resistance.

Although all these assumptions are largely based on logical reasoning, we strongly feel that all future inquiries should be moving in this direction. In addition, the next two steps should be aimed at defining the existence of potential cooperating effects with other approved chemo-drugs and translating the combination of AdipoRon plus gemcitabine into a more complex biological system such as *in vivo* models. Simultaneously, characterizing each mechanistic aspect of the AdipoRon-mediated features may contribute to outlining its wholesomeness in overcoming gemcitabine and, more in general, chemotherapy-induced resistance in PDAC [Figure 1].

In light of the current orphan status and the tremendously unfavorable prognosis, each promising molecule capable of improving both PDAC prognosis and survival should be fully explored. Analyzing the current scientific production, AdipoRon is emerging as a potential therapeutic choice in PDAC, either as a single compound or a partner in gemcitabine-based treatment. Therefore, its pharmacological properties perfectly fulfill the requirements that a candidate is supposed to have for ameliorating PDAC expectation. Whether this is enough to attain clinical approval will be based on the upcoming experiments.

DECLARATIONS

Authors' contributions

Conceptualization: Sapio L, Naviglio S
Investigation: Ragone A, Salzillo A
Writing-original draft preparation: Sapio L
Writing-review and editing: Spina A, Naviglio S

Visualization: Ragone A, Salzillo A
Supervision: Naviglio S
All authors have read and agreed to the published version of the manuscript.

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Cancer Drug Resistance

Commentary

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Ovarian cancer resistance to PARPi and platinum-containing chemotherapy

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Abstract

Epithelial ovarian cancer remains the most lethal female malignancy despite options for systemic therapy and the emergence of targeted therapies. Although initial response to therapy is observed, recurrence and ultimately chemoresistance result in overall therapeutic failure. This pattern has been evident with platinum therapy since the 1980s. Significant excitement surrounded the approval of poly (ADP-ribose) polymerase inhibition (PARPi) as a novel therapeutic option, especially with the advent of personalized medicine, but resistance has similarly developed to these treatments. Novel agents are constantly being sought, but if the obstacle of chemoresistance remains, the durability of responses will remain tenuous. Unraveling the multifactorial mechanisms of platinum and PARPi resistance is increasingly important as a therapeutic failure with current strategies is almost assured. Focusing greater efforts on expanding the current understanding of the complex nature of platinum and PARPi chemoresistance has tremendous potential to improve clinical outcomes.

Keywords: Epithelial ovarian cancer, chemoresistance, platinum resistance, poly (ADP-ribose) polymerase inhibitor resistance, tumor microenvironment

EPITHELIAL OVARIAN CANCER AND THE LIMITATIONS OF CURRENT THERAPY

Epithelial ovarian cancer (EOC), primarily high-grade serous carcinoma (HGSOC), remains the leading

cause of death from gynecologic cancers primarily due to the advanced stage at the time of diagnosis and the inherent difficulty of treating tumors that become resistant to chemotherapy over varying amounts of time. Surgical cytoreduction and platinum-based chemotherapy have been the mainstay of ovarian cancer treatment since the 1980s. Platinum cytotoxicity results from its ability to cause an accumulation of deoxyribonucleic acid (DNA) double-strand breaks and cell death via the formation of intra-strand and inter-strand adducts which activate cellular DNA damage response (DDR) pathways, which inhibit replication and transcription of DNA, inducing cell cycle arrest and apoptosis[1]. Platinum-based therapy has been the cornerstone of EOC treatment for decades, but the majority of patients relapse within two years, and eventual platinum resistance is accompanied by an extremely poor prognosis[2,3]. Platinum-refractory disease is defined as a persistent or progressive disease while receiving platinum-based chemotherapy or within four weeks of therapy, and platinum resistance is defined as cancer recurrence or progression within six months of platinum therapy[3]. The median survival in platinum-resistant disease is only 12 months, underscoring the need to identify novel therapies but also to understand and overcome the complex mechanisms of chemoresistance[4].

The advent of poly (ADP-ribose) polymerase inhibition (PARPi) as a novel therapeutic option approved for EOC in 2014 presented significant excitement and marked the birth of personalized medicine in EOC. Poly (ADP-ribose) polymerases (PARP) are a family of proteins with several roles that include DNA repair (including nucleotide excision repair, non-homologous end joining, homologous recombination (HR) and DNA mismatch repair), apoptosis, chromatin remodeling and the stress response[5]. Several clinical trials have demonstrated the impressive benefit of PARPi therapy, especially in patients who have responded to platinum-based therapy[6,7]. This might be attributable to the high prevalence of tumors with homologous recombination deficiency (HRD), which is found in approximately 50% of all ovarian cancers[6]. BReast CAncer gene (*BRCA1/2*) germline deficiency accounts for approximately 20% of these cases[4]. Somatic BRCA mutations result in HGSOC phenotypes and platinum chemotherapy responses similar to germline BRCA mutation carriers and are referred to as HGSOC with "BRCAness". BRCA variants, either germline or somatic, lead to an increased susceptibility to DNA double-strand breaks. PARPi elegantly capitalizes on synthetic lethality when PARP inhibition and HRD are combined to cause apoptosis of cancer cells[8].

Resistance to platinum therapy is strongly predictive of resistance to PARPi therapy consistent with overlapping biologic mechanisms of susceptibility and resistance[2,9]. A specific definition for PARPi resistance has not yet been developed as exists for platinum-based chemotherapy, and it remains to be seen if tumors are resistant to all PARPi once resistance is developed to a single PARPi[6].

In this commentary, we will explain some of the most clinically relevant tumor biology contributing to platinum and PARPi resistance, two of the most significant therapies in use currently for EOC, as well as review future directions.

MECHANISMS OF CHEMORESISTANCE

There are several mechanisms, both intrinsic and acquired, that contribute to platinum chemoresistance. Platinum compounds may undergo decreased uptake into cells, increased efflux, or increased inactivation. For example, overexpression of the adenosine triphosphate-binding cassette (ABC) family of transporters, such as P-glycoprotein or multidrug resistance-related proteins (MRP1), affects the efflux of chemotherapeutic drugs. Although the exact mechanism of action is not clear, high levels of ABC subfamily A member 1 expression have been found to correlate with poor progression-free and overall survival in serous EOC[10].

Once platinum cytotoxicity has occurred and adducts have begun to form, cells may still be able to thwart its effects by acquiring the ability to repair platinum-induced DNA adducts via counteraction of the cell's DDR response. The DDR response is an essential network of proteins that sense, signal and/or repair DNA damage to maintain genomic integrity and stability within the cell[11]. Key proteins that signal DNA damage to cell-cycle checkpoints and DNA repair pathways include: ataxia-telangiectasia mutated (ATM), ATM- and RAD3-related, DNA-dependent protein kinases[6]. These key proteins sense DNA damage and initiate repair signaling cascades by phosphorylation of key repair proteins: BRCA1, checkpoint kinase 1 (CHK1), checkpoint kinase 2, tumor protein p53 (P53) and *RAD17*[12]. Cellular mechanisms that assist in the repair of DNA include base excision repair for single-strand breaks (SSBs); nucleotide excision repair (NER) for the repair of bulky adducts (such as observed with platinum therapy); mismatch repair (MMR) for mispaired bases; homologous recombination (HR) repair for double-stranded breaks (DSBs), intra-strand and inter-strand crosslinks; non-homologous end-joining (NHEJ) for DSB; or microhomology-mediated end joining for the repair of DSBs[13].

When DNA damage is too severe for repair, apoptosis is triggered[13]. Cells that have acquired platinum resistance subvert the normal DDR responses and instead demonstrate increased ability to initiate repair signaling, activate checkpoints to slow progression and allow repair, increase the pace of adduct repair or even develop the ability to tolerate DNA adducts without resorting to apoptosis[1,14].

The NER and HR repair pathways work very closely together. The NER pathway recognizes and removes bulky helix-distorting crosslink lesions via a step-wise process: damage recognition, unwinding of the DNA locally around damage, incision of damaged DNA by endonucleases, and DNA resynthesis and ligation[15]. This process allows NER to recognize DNA crosslinks and converts the crosslink to a DNA double-strand break. HR is then required to repair the double-strand break to prevent apoptosis[16].

In preclinical trials, enhanced NER in ovarian cancer models has been associated with increased cisplatin resistance *in vitro*, supporting the importance of NER in the development of platinum chemoresistance[17,18,19]. Specifically, the excision repair cross-complementation group 1 (ERCC1) protein plays a key role in nucleotide excision repair. Dimerization of ERCC1 with xeroderma pigmentosum complementation group F (XPF) enables excision and repair of bulky cisplatin lesions in damaged DNA[18]. Overexpression of proteins in the NER pathway, including ERCC1, is associated with platinum resistance, likely via the ERCC1-XPF endonuclease[19,20].

Additionally, P53 expression, common in HGSOC, promotes tumorigenesis as well as drug resistance via inhibition of apoptosis. Inhibited apoptosis contributes to proliferation and metastasis[2]. P53 expression specifically leads to growth phase 1 (G1) / synthesis (S) checkpoint deficiencies and cell-cycle dysregulation, subsequently placing greater stress on the growth phase 2 (G2) / mitosis (M) checkpoint for survival. Wee1 kinase inhibits activation of Cyclin-dependent kinases 1 (CDK1) and CDK2, making it a key cell cycle regulator of the G2/M transition. Alterations in Wee1 kinase may have implications for chemoresistance[21].

Although the majority of EOC responds to platinum therapy, even if only for a limited time, many do not exhibit any durable response to platinum therapy. Mechanisms of such intrinsic chemoresistance include proficient DNA repair, cyclin E1 (CCNE1) amplification, and mesenchymal or proliferative molecular subtypes. Approximately 20% of EOC cases have CCNE1 amplification, which are associated with primary resistance to platinum[22].

The current understanding of the mechanism of PARPi resistance centers around Darwinian escape. Darwinian escape refers to an acquired secondary somatic mutation to restore HR in BRCA 1/2 (reversion mutations)[7]. BRCA reversion mutations have been identified in progression biopsies as well as cell-free DNA (cfDNA). cfDNA has shown polyclonality of multiple reversion mutations within a single patient when under pressure from treatment[8]. One mutation that has demonstrated restoration of DNA repair is *BRCA1-Δ11q*. Patients with *BRCA1-Δ11q* demonstrated decreased sensitivity to both cisplatin and PARPi[23]. BRCA reversion mutations have been identified in 13% of patients with platinum-refractory disease and 18% of patients with platinum-resistant disease[5]. Patients with BRCA reversion mutations experienced decreased progression-free survival when undergoing treatment with rucaparib[24]. The initial strength of response to PARPi has been shown to correlate with the severity of eventual tumor resistance[5].

TUMOR MICROENVIRONMENT AND METABOLISM IMPACT ON CHEMORESISTANCE

Numerous studies have demonstrated the critical role of hypoxia in the tumor microenvironment and its association with platinum resistance via signaling pathways in DNA damage, mitochondrial activity, apoptosis autophagy, and drug efflux[25,26]. Chen *et al.*[27] demonstrated that cisplatin-resistant cancer cells show increased intracellular hypoxia and decreased glucose uptake suggesting that platinum resistance might stem from alterations in glucose metabolism. Altered angiogenesis is another hallmark of intra-tumor vessels which also contributes to hypoxia, poor drug delivery, and chemoresistance[28].

Cancer-associated fibroblasts, tumor-associated macrophages, and cancer stem cells have all been proposed influencers of prognosis and chemotherapy resistance. Stromal activation with extensive desmoplasia has been associated with poor clinical outcomes and acquired treatment resistance[27,29]. Platinum chemotherapy is also thought to influence anti-tumor immunity. Increased infiltration of immunosuppressive Cluster of Differentiation 163-positive macrophages and increased infiltration of regulatory forkhead box P3-positive T cells have favored tumor growth[6,30-32]. In contrast, the presence of tumor-infiltrating lymphocytes is positively correlated with survival[6,33]. Data on immune checkpoint therapy in EOC has been contradictorily reflecting an incomplete understanding of the key regulators and pathways[6]. Immunotherapy may yet play an important role in EOC.

Additionally, the omentum and cell metabolism are thought to play a pivotal role in cancer progression and chemoresistance. The omentum is a favored site for metastasis due to EOC's ability to use fatty acids to initiate and sustain peritoneal metastasis; in addition, it may also participate in ascites formation[34,35]. Elevated cytokines and adipokines in the ascitic fluid are thought to protect malignant cells from chemotherapy-induced apoptosis, including via lysophosphatidic acid signaling and prostaglandin E2 - mediated GFTR transporter upregulation[19]. The metabolism of tumor cells is intricately intertwined with all cells of the microenvironment. Tumor cells have demonstrated an amazing ability to metabolically adjust to changes in their environment, as well as influence the metabolic function of neighboring cells for their own purposes[34,35,36].

Cancer stem cells (CSCs) are theorized as a population of cells capable of self-renewal and repopulation following cancer treatment that may cause tumor initiation and metastasis[37]. Stem cell pathways have recently been recognized as an important mediator of chemoresistance. CSCs have improved methods of chemotherapy removal from the cell, including the ABC cassette and drug transporters. Tumors containing heterogeneous cancer cells are linked to progression as they are more likely to undergo selection towards a population of drug-resistant tumor cells with treatment. CSCs are aided by their microenvironment and stimulated by hypoxia-inducible factors. Phosphoinositide kinase (PI3K) has an important role in apoptosis for maintaining stem cell "stemness" and drug resistance. Notch signaling and Wnt/beta-catenin are

important in CSCs signaling pathways and may be useful targets[38]. CSCs are also able to grow in spheres and expand as sphere-like cellular aggregates more easily than other populations of cells[39]. The ability to treat cancer stem cells is of particular importance since these cells can remain in patients with seemingly no evidence of disease or lead to metastasis.

FUTURE DIRECTIONS

Chemoresistance evolves over time as a response to the selective pressure of therapy. The multiple mechanisms of resistance are complex and not fully understood, but strategies to overcome chemoresistance are being developed. These methods include exploration of methods to improve drug delivery; identification of potentially useful biomarkers; identification of molecular targets; and exploitation of tumor weaknesses using modulation of the cell cycle, tumor microenvironment, or cellular metabolism. Combination therapies may improve this exploitation of tumor weaknesses.

Drug delivery

Improved drug delivery could also combat chemoresistance. Nanotechnology is being used to investigate the ability of nanoparticles to improve targeted treatment, including the encapsulation of cisplatin with dendrimers to aid in cell killing[2,40]. Layer-by-layer nanoparticles have also been used to deliver cisplatin and a PARPi to mice, with improved bioavailability, cytotoxicity, and systemic toxicity compared with conventionally administered medication[40]. Natural compounds including curcumin and piperine are being evaluated to attempt to induce G2/M phase arrest, caspase activation, the PI3K pathway (piperine), and apoptosis and phosphorylation of p53 (curcumin)[2].

Biomarkers

A study of patients with durable responses may be useful for identifying biomarkers, especially patients with HR proficiency who have good responses[7]. Improved understanding of why some patients with BRCA pathogenic variants do not respond to PARPi, and why some patients with no known BRCA mutation respond to PARPi treatment could be important for combatting drug resistance. Biomarkers that predict treatment success would be of significant benefit if they allowed improved treatment selection. However, our understanding of biomarkers that would be useful in guiding treatment selection is limited. A number of genes and proteins have been identified in patients with chemoresistance or poor prognosis and have yet to be evaluated in clinical trials to guide treatment. One gene amplification, 19q12, is associated with chemoresistance and has been identified in 20% of ovarian cancer patients[3,40]. MMR deficiency has also been implicated in chemoresistance, but the correlation is controversial[3,19,41].

Based on the leading roles played by ERCC1 and XPF in the NER pathway, they have been promising prospects as biomarkers for platinum-based therapy. A meta-analysis of retrospective *in vitro* studies in non-small cell lung cancer by Altaha *et al.*[42] concluded that high levels of ERCC1-mRNA and/or ERCC1 protein were associated with resistance to platinum compounds. Despite the correlation between ERCC1 overexpression with platinum resistance and ERCC1 under expression with platinum sensitivity, translating ERCC1 as a predictive biomarker for response to platinum-based therapy in clinical trials has been challenging[43]. ERCC1 has been investigated as a biomarker in both adrenocortical carcinomas and advanced non-small cell lung cancer and did not offer prognostic or treatment selection benefits[44,45]. Continued study of ERCC1 and the NER pathway is needed.

Patients with PARPi resistance have been found to have reversions in *RAD51C* and *RAD51D*, and in vitro studies have demonstrated loss of TP53 binding protein 1[3,46]. Studies of TP53 vary regarding its utility as a marker in platinum resistance, but it may be helpful in treatment planning[47]. Pump P-glycoprotein (P-gp)

expression, which is related to drug efflux and multidrug resistance, has also been implicated in PARPi resistance[3,48]. CSC markers of *Bmi-1*, *Nanog* and *Oct 4* with elevated stem cell factor and *c-Kit* levels indicated chemoresistance as well[37]. Other proteins indicating resistance and poor prognosis include Notch3 and *LGR5*; CD24 correlates with poor prognosis, chemoresistance, metastasis, and recurrence; CD44+/CD117+ cells were associated with chemoresistance; CD133+ cells showed cisplatin resistance, and ALDH1A1 was associated with poor survival and drug resistance[36,49]. AT-rich interaction domain 1A inactivation or loss of expression is also correlated with poor overall survival in patients receiving platinum. Micro ribonucleic acids (miRNAs), including let-7 g, miR-98-5p, miR-622 as well as others, have been associated with platinum and/or PARPi resistance[50].

Newer technology in tumor surveillance may also be critical to the understanding of tumor chemoresistance and the discovery of biomarkers. Liquid biopsies may offer a unique opportunity for real-time tumor molecular profiling. Liquid biopsies enable the analysis of circulating tumor cells, circulating tumor DNA, circulating mRNA, and tumor-derived extracellular vesicles that are shed from primary tumors into the peripheral blood[51]. Serial assessments would enable real-time patient assessment and comparison during therapy at the onset of resistance to therapy, which may increase our understanding of chemoresistance.

Genetics

Other possible areas of investigation involve the utilization of next-generation sequencing to aid the discovery of predictive signatures. Four molecular subtypes of HGSOC have been described and validated by the Cancer Genome Atlas Research Network study[52]. Absolute copy number profiles have been generated using primary and relapsed HGSC samples. Macintyre *et al.*[53] identified seven copy number signatures that were stable between diagnosis and disease relapse, indicating that copy number signatures at diagnosis may predict overall survival and platinum resistance[54]. Different mutational processes generate unique combinations of mutation types, termed mutational signatures[55]. The Catalogue of Somatic Mutations in Cancer (COSMIC) has revealed many mutational signatures across the spectrum of human cancer types. Analysis of COSMIC mutational signatures in ovarian cancer reveals a prevalence of signature 1B[56].

Epigenetic modifications are mechanisms that alter the expression of a gene but do not change the DNA sequence. These modifications help to regulate normal genome functioning. Epigenetic modifications may also be contributing to chemoresistance. Identification of epigenetic changes in chemoresistant EOC may allow for the advanced development of epigenetic modulating drug therapy. Current agents such as demethylating agents, histone deacetylase inhibitors, and microRNA-targeting therapies have shown preclinical promise[57].

Homologous recombination and combination therapies

Defining PARPi resistance and evaluating the utility of alternating PARPi therapy if resistance has developed to one PARP remains to be seen but may be answered by the OReO trial[6,58]. PARPi do have different potencies due to the degree of PARP trapping; clinical implications have yet to be seen. Additionally, re-examining the definition of platinum sensitivity and resistance may be needed.

Combination therapies of PARPi and platinum together, or the addition of another agent to combat chemoresistance when using one or the other, may allow circumvention of treatment resistance. In a study by Gajan *et al.*[58] of breast cancer patients with and without BRCA mutations and with resistance to both cisplatin and olaparib, a benefit of combined therapy with olaparib and cisplatin was identified. Interestingly, the combination treatment was not helpful in patients whose disease was still sensitive to either treatment[59]. A similar investigation could be considered in EOC patients as well. Niraparib has been

investigated in combination with carboplatin and gemcitabine for patients with unresectable or recurrent platinum-sensitive ovarian cancer and showed a 57% radiographic response rate[60]. Cisplatin-resistant ovarian cancer cells (C13) were treated with a PARPi, 3-aminobenzamide by Zhang *et al.*[60] Cellular proliferation was inhibited more when treated with cisplatin and increasing doses of PARPi, pointing to chemosensitization[61]. Additional studies investigating novel DDR-targeting agents and combination treatment approaches are underway.

As targetable pathways have been implicated in chemoresistance, translational researchers have also been searching for immunotherapies and molecular targets to use as combination therapies to improve the cytotoxic effect of therapy. Anti-angiogenesis with cediranib in combination with olaparib has been investigated with efficacy that varied by mutation identified following PARPi progression, with poorer outcomes identified for patients with HRD reversion mutations and ABCB1 upregulation[62]. This treatment combination is under further investigation with the OVC2 trial[6,63]. One promising target for therapeutics to combine with PARPi is mesenchymal-epithelial transition factor (c-Met), a receptor tyrosine kinase, since the high expression is associated with a poor prognosis. This biomarker and therapeutic target have been linked to PARP resistance and HR restoration[7]. CDK have also been proposed as targets since they control cell cycle progression and DNA damage control. Loss of CDK inhibition impairs BRCA function (BRCAness)[7]. PI3K inhibitors have been proposed as methods to extend the use of PARPi and are under investigation[7,64,65]. Other therapies that have been trialed include PARPi with alkylating agents, topoisomerase I inhibitors, WEE1 kinase inhibitors, PI3K inhibitors, radiation, immunotherapy, and DNA methyltransferase inhibitors. PARPi with topoisomerase I inhibitor allows inhibition of TOP1-PARylation, inhibition of homologous recombination and stimulation of NHEJ plus inhibition of tyrosyl-DNA-phosphodiesterase I. Clinical investigations of this combination have not been completed[5]. However, promising combination treatments to overcome PARPi or platinum resistance have not been identified.

CONCLUSIONS

EOC is a disease marked by frequent relapses and responses to therapy before the eventual development of chemoresistance. Chemoresistance remains a steadfast obstacle to improving EOC survival. Platinum, the mainstay of EOC treatment, is limited by almost ubiquitous platinum resistance. While anti-angiogenic therapy and PARPi are promising new treatments, resistance has already been observed. The heterogeneity of EOC allows it to adapt in response to treatment and confounds attempts to identify clinically meaningful biomarkers and targets. Tumors constantly evolving with time and treatment seem to naturally develop resistance.

In some cancers, real-time collection of circulating tumor DNA is a reality that has enabled the characterization of drug response for tumors along with multiple time points[62]. EOC, given its inherent heterogeneity, may require an equally malleable treatment approach: the serial integration of genomic, epigenomic, metabolomic, and immunogenic study at diagnosis and at each recurrence to optimize therapy. It would require a significant change in clinical practice and greater investment in bioinformatics, but it may not be possible to conquer all the nuances of EOC without it.

DECLARATIONS

Authors' contributions

Made substantial contributions to the conception and design of this study, performed literature review, wrote and edited the manuscript: Summey R

Conceived and designed this study, performed literature review, co-wrote and edited the manuscript: Uyar D

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Cancer Drug Resistance

Review

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Therapeutics to harness the immune microenvironment in multiple myeloma

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Abstract

Multiple myeloma (MM) remains an incurable, genetically heterogeneous disease characterized by the uncontrolled proliferation of transformed plasma cells nurtured within a permissive bone marrow (BM) microenvironment. Current therapies leverage the unique biology of MM cells and target the immune microenvironment that drives tumor growth and facilitates immune evasion. Proteasome inhibitors and immunomodulatory drugs were initially introduced to complement and have now supplanted cytotoxic chemotherapy as frontline anti-myeloma agents. Recently, monoclonal antibodies, bispecific antibodies, and chimeric antigen receptor T cells were developed to revamp the immune system to overcome immune suppression and improve patient responses. While current MM therapies have markedly extended patient survival, acquired drug resistance inevitably emerges and drives disease progression. The logical progression for the next generation of MM therapies would be to design and validate agents that prevent and/or overcome acquired resistance to immunotherapies. The complex BM microenvironment promotes resistance to both current anti-myeloma agents and emerging immunotherapies. Myeloma cells are intertwined with a complex BM immune microenvironment that contributes to the development of adaptive drug resistance. Here, we describe recently FDA-approved and investigational anti-myeloma agents that directly or indirectly target the BM microenvironment to prevent or overcome drug resistance. Synergistic effects of anti-myeloma agents may foster the development of rationally-designed drug cocktails that prevent BM-mediated resistance to immunotherapies.

Keywords: Multiple myeloma, drug resistance, proteasome inhibitors, immunomodulators, immunotherapeutics, adaptive resistance, bone marrow microenvironment

MULTIPLE MYELOMA

Multiple myeloma (MM) is described by clonally expanding plasma cells within the bone marrow (BM), monoclonal proteins detected in blood or urine, and end-organ damage[1,2]. Approximately 13% of all hematologic cancers are classified as MM, which is the 2nd most common hematological cancer in high-income and Western countries[3]. In the US in 2021, approximately 34,920 new cases of MM (19,320 men, 15,600 women) were reported[3-6].The lifetime risk of an MM diagnosis is 1 in 125 (0.8%). The annual incidence of MM/100,000 persons is 8.2 cases (Caucasian men), 5.0 cases (Caucasian women), 16.5 cases (African-American men), 12.0 cases (African-American women), 8.2 (Hispanic men), 5.7 (Hispanic women) and 5.0 (Asians/Pacific Islander men), and 3.2 (Asians/Pacific Islander women)[3-6]. Approximately 12,410 deaths from MM (6,840 men, 5,570 women) were expected in 2021[4]. Newly reported cases of MM did not change significantly over the past 10 years, staying in the range of 6.7/100,000 since 2010, while death rates declined slightly, from 3.4/100,000 in 2008 to 3.1/100,000 in 2018[4]. Expected 5-year survival has improved to ~56%[3-6]. Risk factors include obesity, chronic inflammation, exposure to pesticides, organic solvents, and radiation, and inherited genetic variants[6-8].

MM starts as an asymptomatic precursor condition monoclonal gammopathy of undetermined significance (MGUS) or smoldering multiple myeloma (SMM). Genetic abnormalities, e.g., hyperdiploidy, translocations are already evident in MGUS and SMM[9-11]. While these precursors may exhibit a significant burden of clonal plasma cells, they require additional genetic changes to drive end-organ damage and become MM.

Current MM therapy leverages the unique features of plasma cell biology that proliferate within the BM to promote deep clinical remissions with fewer side effects than cytotoxic chemotherapy. The first FDA-approved proteasome inhibitor (PI) bortezomib, immunomodulatory drugs (IMiDs) thalidomide, lenalidomide and pomalidomide (Celgene), and monoclonal antibodies that target CD38 and SLAMF7 have significantly extended patient outcome [Table 1][9,12,13]. These FDA-approved agents are used to treat newly diagnosed patients with related next-generation agents exhibiting activity in the relapsed and/or refractory MM (RRMM) in all stages of treatment[9,12,13]. While these agents have markedly improved survival, MM remains incurable, with therapeutic resistance invariably emerging even in patients with an initial favorable response to therapy. Further efforts are needed to define tumor and BM-driven resistance mechanisms to inform next-generation therapies.

MM EFFECTS ON BONE MARROW

Evasion and suppression of antitumor immunity is an essential step in myelomagenesis. MM cells replicate and proliferate nearly exclusively within the BM niche, highlighting the role of the microenvironment in supporting cancer growth[14-16]. The BM microenvironment is highly vascularized and consists of a cellular compartment divided into hematopoietic cells, e.g., hematopoietic stem cells (HSC), T and B lymphocytes, myeloid and natural killer (NK) cells, and osteoclasts. The non-hematopoietic cell types include bone marrow stromal cells (BMSCs), osteoblasts, endothelial cells (EC), and fibroblasts[13-16]. The non-cellular compartment consists of an extracellular matrix (ECM), oxygen concentration, and a soluble cocktail milieu of growth factors, cytokines and chemokines. Plasma cell clones traffic in and out of the BM to foster metastatic progression while other cell types re-circulate into and out of the BM to promote cytokine-driven myeloma growth[14-16]. The tumor-immune microenvironment supports the acquisition of resistance to

Table 1. Effects of FDA-approved and investigational agents on the myeloma immune microenvironment

Therapeutic agent	Stage	Target	Effect on myeloma-immune microenvironment
Proteasome inhibitors			
Bortezomib (Velcade)	FDA-approved	Proteasome 5	Bortezomib inhibits osteoclast differentiation induced by the RANKL, stimulates osteoblast differentiation and inhibits autocrine/paracrine signaling in MSCs and in ECM. PIs also reduce MM adhesion to BMSCs[15,16,17,24,33-35]
Carfilzomib (Kyprolis)	FDA-approved	Proteasome 5	
Ixazomib (Ninlaro)	FDA-approved	Proteasome 5	
IMiDs			
Thalidomide (Thalomid)	FDA-approved	CRBN	IMiDs promote anti-proliferative, T-cell co-stimulatory, anti-angiogenic and anti-inflammatory effects[38-44]
Lenalidomide (Revlimid)	FDA-approved	CRBN	
Pomalidomide (Pomalyst)	FDA-approved	CRBN	
Cel-MODCC-92480	Phase 1/2	CRBN E3 Ub ligase Modulator	Antitumor and immunostimulatory activities
CC-220 (Iberdomide)	Phase 1b/2a	CRBN E3 Ub ligase Modulator (CelMOD)	Antitumor and immunostimulatory activities
C-92480 (Mezigdomide)	Phase 1/2	CRBN E3 Ub ligase Modulator (CelMOD)	Antitumor and immunostimulatory activities
Monoclonal antibodies			
Daratumumab (Darzalex)	FDA-approved	CD38	Augments NK-cell cytotoxicity, induces robust increases in helper and cytotoxic T-cell absolute counts. Increases memory T cells while decreasing naïve T cells. Eliminates CD38+ immune suppressor cells, e.g., Tregs, Bregs, and MDSCs[61-69]
Elotuzumab (Empliciti)	FDA-approved	SLAMF7	Induces TAM activation and mediates ADCP through an FcγR-dependent manner *in vitro*
Isatuximab (Sarclisa)	FDA-approved	CD38	Eliminates CD38+ immunosuppressive Tregs and alleviates BM-induced immunosuppression
Nuclear export inhibitors			
Selinexor (Xpovio)	FDA-approved	Exportin 1, (XPO)	Increased NK cell cytotoxicity in vitro, potentiates ADCC, downregulates pro-survival signals from BM microenvironment, blunts the protective effects from pro-survival signals from TNF, IL-6, IL-4, BAFF BMSCs[82-87]
Bone-modifying agent			
Denosumab (Prolia)	FDA-approved	RANKL	Potent Inhibitor of osteoclast function[88-90]
Bisphosphonates			
Zoledronate (Zometa)	FDA-approved	Farnesyl diphosphate	Reduces osteoclast function, inhibits liberation of matrix-synthase (FDPS) bound cytokines, increases IFN-γ production by IL-2-primed NK cells, decrease tumor cell adhesion to bone, and activates T cells[91-94]
CAR T cells			
Idecabtagene vicleucel (ida-cel, Abecma, bb2121)	FDA-approved	TNFRSF17 (BCMA)	T cells are physically recruited and linked to tumor surface Ags to elicit an anti-tumor immune response and overcome BM microenvironment-mediated immunosuppression[95-101]
Ciltacabtagene autoleucel (Carvykti, cilta-cel LCAR-B38M, JNJ-4528)	FDA-approved	Two llama-derived Abs that bind human BCMA	Reduce BCMA-cell expression and microenvironment-mediated immunosuppression

Bispecific T Cell Engagers			
Blinatumumab (Blincyto)	FDA-Approved (R/R ALL) Pilot Study (MM) (NCT03173430)	CD19-targeting engager (CD19xCD3)	BiTEs bind simultaneously to T cells and tumor Ags, recruits T cells to tumors and tumor T-cell microenvironment, leading to T cell activation, proliferation, and tumor cell death
Talquetamab	Phase I/II (MM) (NCT03399799)	GPRC5D-targeting bi-specific T-cell engager (GPRC5D x CD3)	Actively kills GPRC5D+ MM cell lines and primary MM cells *in vitro*[110-115,121]
AMG420 (NCT03836053)	Phase 1b (RRMM)	BCMA-targeting bi-specific T-cell engager (BCMA x CD3)	Short half-life with encouraging activity in RRMM. Three patients dosed with 400 μg/d had MRD-negative CRs, 2 more responders in the dose confirmation cohort, 3 patients at lower doses attained CRs. No major toxicities were observed up to 400 μg/d[110-115,122,123]
Teclistamab (JNJ-64007957)	Phase 1 (MM) (NCT03145181)	BCMA-targeting bi-specific T-cell engager (BCMA x CD3)	At the phase 2 dose, showed promising efficacy and durable responses, well tolerated[110-115,124]

Effect of FDA-approved and developmental agents on other cell types within the BM microenvironment. Listed are anti-myeloma agents, putative targets and effects within the myeloma microenvironment. FDA: Federal Drug Administration; IMiD: immunomodulatory drug; NF-κB: nuclear factor kappa-B; MSC: mesenchymal stem cell; ECM: extracellular matrix; BMSC: bone marrow stromal cell; CRBN: Cereblon (CUL4-CRBN E3 Ub ligase complex); Ub: ubiquitin; TAM: tumor-associated macrophage; RANKL: receptor activator of NF-κB ligand; RRMM: relapsed and/or refractory multiple myeloma; BiTE: bispecific T cell engager; SLAMF7: signaling lymphocytic activation molecule F7; TNF: tumor necrosis factor; IFN-γ: interferon-gamma; BAFF: B-cell activating factor; MDSCs: myeloid-derived suppressor cell; ADCP: antibody-dependent cell cytotoxicity; ALL: acute lymphoblastic leukemia; Ags: antigens; BCMA: B-cell maturation antigen; GPRC5D: G protein-coupled receptor, class C group 5 member D.

cytotoxic chemotherapy, biologic agents and immunotherapies leading to immune escape[17-20]. Recently it was shown using a pumpless culture platform that adhesion of patient-derived MM cells (PMMCs) to osteoblasts and osteoblast long-term viability were critical factors for ex vivo survival of PMMCs[21]. Osteoblasts can also subvert the anti-myeloma effect of NK cells. Since NK cells (and genetically-engineered chimeric antigen receptor-modified NKs) have clinical potential, a better understanding of the osteoblast role as immune regulators in BM is essential[22]. Similarly, osteoclasts regulate antigen-dependent T cell activation and responses. Like macrophages, monocytes, and dendritic cells (DCs), osteoclasts display phenotypic and functional plasticity that is dependent on their origin and environment[23].

The highly organized BM integrity is disrupted by the invasion of MM cells. A liquid milieu of cytokines, chemokines, growth factors, and inflammatory mediators mixed with matrix remodeling enzymes enables the communication between tumor, immune and microenvironment cells. Circulating tumor cells, exosomes, cell-free DNA, and apoptotic bodies negotiate the transfer of genetic information from myeloma cells to other tumor and non-tumor cell types[24-29]. Exosomes are small, secreted vesicles that confer the bidirectional transfer of proteins, lipids, and nucleic acids between BM and tumor cells. Exosomes can support myelomagenesis by promoting angiogenesis, osteolytic lesions, and drug resistance[17-21]. The content of exosomes from MM patients differs from that of healthy donors and could potentially serve as biomarkers and targets.

MM cells are decorated with adhesion molecules that localize myeloma cells to the ECM[30]. Collagen I, collagen III, and elastin recently were shown to block the cytotoxic effect of NK cells and promote their production of chemokines and cytokines[31]. NK cell cytotoxicity against major histocompatibility complex (MHC)-I-deficient melanoma was markedly increased by blocking tumor collagen deposition. MHC-I down-regulation occurred in solid cancers, which could be directly targeted by circulating cytotoxic NK cells. Prior studies have demonstrated that BMSCs produce paracrine factors and cytokines that drive cell-cell engagement and induce the generation of osteolytic lesions[32-38]. Physical interaction between MM and BMSCs, as well as transforming growth factor (TGF) and interleukin (IL)-6 enhance the formation of lytic bone lesions[32,33]. Cell-cell interactions and cell adhesion also enhance drug resistance in MM cells[33-36]. Unlike healthy mesenchymal stem cells (MSCs), myeloma MSCs enhance tumor survival by producing elevated levels of IL-1β and tumor necrosis factor-alpha (TNF-α)[33-39].

PROTEASOME INHIBITORS

Proteasome inhibitors (PIs) are the backbone components of current anti-myeloma regimens[40]. Bortezomib (Millennium-Takeda) demonstrates clinical efficacy and safety for newly diagnosed and RRMM disease. However, the emergence of chemoresistance and the development of adverse effects, especially peripheral neuropathy, can limit clinical utility. Second-generation PIs carfilzomib (Onyx/Amgen) and ixazomib (Millennium-Takeda) are approved for RRMM and may overcome resistance with better tolerability. Bortezomib has received regulatory approval for intravenous and subcutaneous administration, while ixazomib is the only orally bioavailable PI.

PIs also target components of the BM immune microenvironment [Figure 1][40,41]. Kim *et al.* reported that bortezomib impaired BMSCs proliferation *in vitro*[41]. PIs downregulate autocrine and paracrine signaling signaled by ECM and MSCs, which impairs myeloma cell growth and survival[14,42]. In addition, PIs suppress interleukin-6 (IL-6), IGF-1, and TNF-α production to decrease CXCL12 production by BMSCs[43,44]. BM angiogenesis also plays an important role in myelomagenesis and suppresses angiogenesis by decreasing VEGF secretion. Roccaro *et al.* studied MM patient-derived endothelial cells to determine the effects of bortezomib on the angiogenic phenotype[43]. At clinically achievable doses, bortezomib inhibited the proliferation of MM patient endothelial cells as well as human umbilical vein endothelial cells in a dose- and time-dependent manner. The binding of MM.1S cells to patient-derived endothelial cells augmented the proliferation of myeloma cells, which was abolished by bortezomib. Bortezomib blocked vascular endothelial growth factor (VEGF) and IL-6 secretion by endothelial cells from myeloma patients and reduced VEGF, IL-6, insulin-like growth factor-I, Angiopoietin (Ang1/Ang2) transcription. Taken together, the results illustrate that bortezomib elicits anti-angiogenic effects in BM.

IMMUNOMODULATORY DRUGS

Thalidomide, lenalidomide, and pomalidomide (Celgene/Bristol-Myers Squibb) are immunomodulatory drugs (IMiDs) that have contributed to the improvement in MM patient survival[44]. Lenalidomide is employed to treat transplant-eligible and ineligible (NDMM) as maintenance post-transplant and for RRMM. IMiDs are thalidomide analogs, which exhibit pleiotropic anti-myeloma activities such as anti-proliferation, anti-angiogenesis, anti-inflammatory, immunomodulatory and cytotoxic effects[44-47]. IMiDs also impact the BM microenvironment to lower IL-6 concentrations. Following the introduction of alkylating agents for MM, thalidomide was the next agent to change disease course through VEGF suppression, immunomodulatory and anti-inflammatory effects[44-46]. IMiDs increase T cell and NK cell activity, downregulate cytokines, inhibit bone resorption, and decrease cell adhesion molecules (CAM) to disrupt MM-BMSC interactions and IL-6 production[45-47].

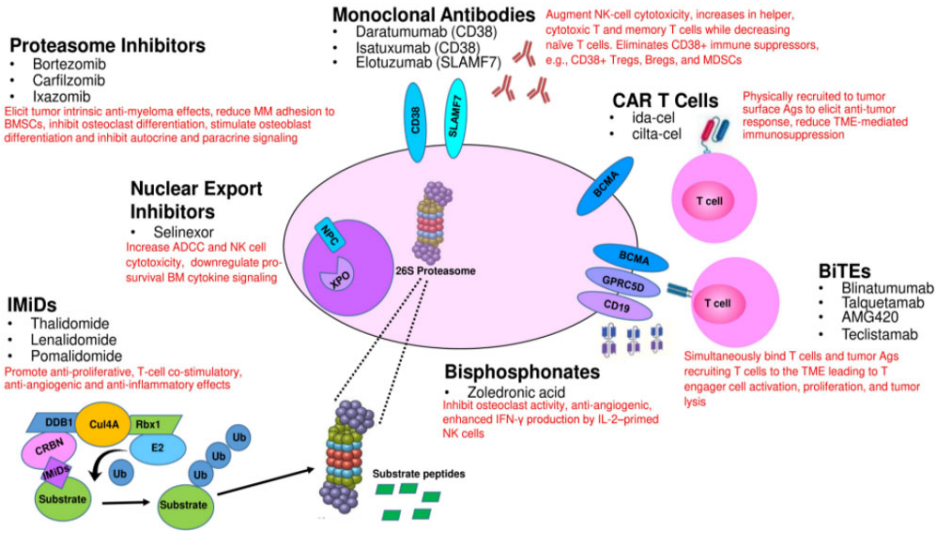

Figure 1. Direct and indirect effects of FDA-approved and developmental agents on MM cells and their interaction with other cell types within the tumor microenvironment. CAR: Chimeric antigen receptor.

Communication between myeloma cells and other cellular components of the tumor microenvironment, e.g., osteoclasts, osteoblasts, and BMSCs, is bidirectional and highly complex. Lenalidomide downregulates hyperactive osteoclasts and reduces the secretion of osteoclastogenic MIP-1a, B-cell activating factor (BAFF), a proliferation-inducing ligand (APRIL), and receptor activator of nuclear factor kappa-B (NF-κB) ligand (RANK-L)[48,49]. Lenalidomide has also been shown to more significantly decrease TNF-α, IL-1β, IL-6, and interleukin-12 (IL-12) levels and increases interleukin-2 (IL-2) and IFN-γ production compared to thalidomide[50]. LeBlanc *et al.* found that IMiDs co-stimulated T cells through the B7-CD28 pathway[51]. IMiDs prolonged T cell priming and boosted the uptake of tumor antigens by DCs to improve the efficacy of antigen presentation[52,53]. IMiDs also enhance NK and NK T cell activities[54] and inhibit T regulatory cells (Tregs) proliferation and activity[55]. IMiDs decrease IL-2, IFN-γ, and SOCS1 expression in CD4+ T, CD8+ T, NK+ T, and NK cells from peripheral blood (PB) and B[56].

Programmed death (PD)-1 and PD-ligand-1 (PD-L1) interactions attenuate the production of cytotoxic T lymphocytes (CTLs) that recognize tumor cells. PD-L1 expression on plasma cells from MM patients is markedly upregulated compared to those from MGUS patients and healthy volunteers[57]. IMiDs downregulate PD-1 levels on T and NK cells and PD-L1 on myeloma cells to promote antibody-dependent cellular cytotoxicity (ADCC). Bortezomib and lenalidomide do not have the flexibility to subdue myeloid-derived suppressor cell (MDSC) activity, whereas CD38-targeting agents do have this capacity[58,59]. Co-stimulatory effects of IMiDs on T and NK cells have been proposed to enhance anti-MM immunity but are yet to be demonstrated *in vivo*.

MONOCLONAL ANTIBODIES

The introduction of PIs and IMiDs represented an initial paradigm shift in MM treatment strategy. Subsequently, in 2015 two monoclonal antibodies were FDA-approved for RRMM treatment and represented a second shift in the treatment approach towards immunotherapies. Daratumumab (Janssen Oncology) is a humanized monoclonal IgG-κ antibody that binds to the transmembrane glycoprotein CD38 (cyclic ADP ribose hydrolase)[60]. CD38 is expressed on immune cells, overexpressed on myeloma cells, and contributes to cell adhesion and ecto-enzymatic activities. Daratumumab binds CD38, causing cells to undergo ADCC, complement-dependent cytotoxicity (CDC), and antibody-dependent cellular phagocytosis

(ADCP). Combination regimens incorporating daratumumab have demonstrated promising results in the relapsed refractory setting and are increasingly used upfront and in transplant-eligible patients[61-64]. Phase III trials showed promising results when daratumumab was combined with lenalidomide and dexamethasone, with bortezomib and dexamethasone, and, in quadruplet therapy with bortezomib, dexamethasone, and lenalidomide[61-64].

Daratumumab also targets CD38+ immune, non-myeloma cell populations. PB and BM were obtained and analyzed before and during therapy and at relapse from RRMM patients enrolled in two daratumumab monotherapy studies[65]. CD38-expressing regulatory B cells (Bregs) and MDSCS were evaluated to determine the effect of daratumumab on immunosuppressive activity. A unique subpopulation of CD38+ Tregs was found to be more immunosuppressive than CD38- Tregs *in vitro* and was reduced in daratumumab-treated patients. Likewise, daratumumab treatment generated significant elevations in helper and cytotoxic T-cell absolute counts. In PB and BM, daratumumab induced significant increases in CD8+:CD4+ and CD8+:Treg ratios and increased memory T cells while decreasing naïve T cells. The majority of patients demonstrated broad T-cell changes, although patients with a partial response or better showed greater maximum effector and helper T-cell increases. Greater increases in T-cell clonality, measured by T-cell receptor (TCR) sequencing, positively correlated with increased CD8+ PB T-cell counts. Depletion of CD38+ immunosuppressive cells, which is related to a rise in T-helper cells, cytotoxic T cells, T-cell functional response, and TCR clonality, represents an additional mechanism of action for daratumumab and deserves further exploration. The anti-myeloma benefit of daratumumab can be potentiated when combined with bortezomib which leads to increased expression of CD38 target on MM cells. However, daratumumab may also internalize CD38 in MM cells to inhibit adhesion to BMSCs and overcome CAM drug resistance (CAM-DR)[66].

The second humanized monoclonal FDA-approved for MM is elotuzumab (Bristol-Myers Squibb), which binds the signaling lymphocytic activation molecule family 7 (SLAMF7, CD319, cell-surface glycoprotein CD2 subset 1/CS1), on the MM cell surface[67]. SLAMF7 is also modestly expressed on NK cells and certain T cells[68,69]. In combination with lenalidomide and dexamethasone, elotuzumab enhances progression-free survival (PFS) in RRMM.

Awwad *et al*. demonstrated that SLAMF7 was expressed at high levels on CD8+CD28-CD57+ Tregs from MM patients[70]. SLAMF7 levels were also linked with the expression of T cell exhaustion transcription factor signatures and cell surface markers. Elotuzumab specifically depleted SLAMF7+CD8+ T cells *in vitro* and *in vivo* through macrophage-dependent ADCP. SLAMF7 may serve as an indicator to identify CD8+ Tregs and anti-SLAMF7 antibodies that enhance anti-myeloma responses.

Isatuximab (Sanofi/Genzyme) targets a specific epitope on the transmembrane glycoprotein CD38, different from that targeted by daratumumab, and inhibits CD38 hydrolase activity[68-75]. CD38 regulates migration and receptor-mediated adhesion by binding to CD31 or hyaluronic acid. Isatuximab induces myeloma death through fragment crystallizable (Fc)-dependent mechanisms, e.g., ADCC, ADCP, and CDC, and direct Fc-independent mechanisms[72]. Isatuximab downregulates constitutive and inducible Tregs resulting in enhancing the anti-myeloma response of other immune cell types[73-75].

Isatuximab was evaluated as monotherapy and demonstrated promising results in a phase I study of 35 RRMM patients as well as a subsequent phase II study alone and in combination with dexamethasone in heavily-pretreated patients[76,77]. Isatuximab added to a pomalidomide-dexamethasone regimen improved PFS, which represents another option to treat lenalidomide- and PI-refractory disease[78]. When combined

with pomalidomide and low-dose dexamethasone, isatuximab led to improved PFS and a 40% reduction in the risk of disease progression or death. Patients had an overall response rate (ORR) of 60.4%, compared to 35.3% for patients who only received pomalidomide and dexamethasone. Isatuximab demonstrated a statistically significant increase in TCR clonality after treatment compared to that at treatment initiation, suggesting that isatuximab increases host antitumor immunity.

A recent study assessed isatuximab in heavily-pretreated RRMM patients despite receiving prior anti-CD38 therapy, most patients having been recently exposed to daratumumab combination therapy[79]. Most patients (77%) experienced a response of MR or better with isatuximab. While objective responses were not observed, one patient achieved MR and 17 patients had stable disease as the best overall response[80]. A prospective, randomized, open-label, phase 3 trial compared isatuximab combined with carfilzomib-dexamethasone to carfilzomib-dexamethasone in relapsed MM patients[81]. Isatuximab addition significantly improved PFS and depth of response, representing a new standard of care for this group.

Daratumumab, elotuzumab, and isatuximab act by recruiting immune effectors to enhance cellular cytotoxicity directed against myeloma cells. The anti-myeloma activity of daratumumab and elotuzumab appears independent of the disease stage. These agents may adversely generate allergic-type infusion reactions. Potential complications in serum protein electrophoresis testing and daratumumab cross-reactivity with CD38 present on erythrocytes should be considered. The success of daratumumab and elotuzumab in RRMM has ignited enthusiasm for the development of additional CD38-targeting agents. To note, hypoxia within the BM microenvironment suppresses the maturation of MM cells as well as the expression of CD38 and SLAMF7. While antibody therapy was initially approved for RRMM, there is interest in incorporating monoclonal antibodies into conditioning regimens for NDMM as well.

NUCLEAR EXPORT INHIBITORS

Selinexor (Karyopharm Therapeutics) is a first-in-class orally bioavailable drug that inhibits the nuclear export protein exportin1 (XPO1)[82]. Selinexor has been FDA-approved for use combined with dexamethasone and bortezomib in MM patients previously treated with four prior therapies, including at least two PIs and at least two IMiDs[82,83]. XPO1 overexpression is linked with a worse prognosis in solid tumors and blood cancers[84]. Selinexor also demonstrates an ability to modulate tumor immunology and the surrounding tumor microenvironment. Treatment of B cell lymphomas with selinexor led to increased NK cell-mediated cytotoxicity *in vitro* and selinexor potentiated ADCC-mediated by rituximab and obinutuzumab[85]. NK cells exhibited greater IFN-γ and CD107a expression, both activities associated with NK activation, and lymphoma cells downregulated HLA-E, which binds the inhibitory NKG2A receptor. Zhong *et al.* demonstrated that selinexor may also downregulate pro-survival signals that originate from the BM microenvironment[86]. Treatment of CLL cells with selinexor blunted protective effects from anti-apoptotic, pro-survival signals from TNF, IL-6, interleukin-4 (IL-4), BAFF, and CD40L *in vitro* and also blunted anti-apoptotic effects of marrow-derived fibroblast co-culture model. Selinexor may help overcome hypoxia-mediated PI resistance *in vitro* as well and restore PI sensitivity *in vivo*[87].

DENOSUMAB

The receptor activator of nuclear factor-κB ligand (RANKL)/RANK signaling system modulates osteoclastogenesis leading to bone resorption[88]. Denosumab (Amgen) is a humanized monoclonal antibody that neutralizes RANKL, inhibits osteoclasts, and decreases the rate of skeletal-related events not only in MM but also in solid tumors[89,90]. Denosumab treatment can inhibit the RANKL/RANL receptor interaction and suppress osteoclastic bone resorption.

BISPHOSPHONATES

Zoledronate (Novartis) and pamidronate (Novartis) are pyrophosphate analogs (bisphosphonates), which demonstrate a high affinity for bone and the capacity to impair osteoclast function as well as anti-angiogenic activities[91-93]. Bisphosphonates inhibit farnesyl pyrophosphate synthase and reduce isoprenylation of Rab, Rac, and Rho. The bisphosphonates are provided as supportive therapy in MM since they are associated with lower rates of vertebral fractures, reduced skeletal-related events, and decreased pain but are associated with an increased risk of jaw osteonecrosis. Nussbaumer *et al.* showed that zoledronic acid enhanced IFN-γ production by IL-2-primed NK cells in CD14+CD56+ DC-like cell-dependent process that may also require γδ T cells[94].

CAR T CELLS AND BISPECIFIC T CELL ENGAGERS

The adoptive transfer of chimeric antigen receptor (CAR)-expressing T cells is a transformative approach to improve cancer treatment. B-cell maturation antigen (BCMA) displays restricted RNA expression and is selectively expressed by B-lineage cells. BCMA is not detected in healthy tissues and was not detected on human CD34+ HSCs[95]. T cells expressing a CAR-targeting BCMA had substantial activity against heavily-treated RRMM patients[96]. BCMA-targeted CAR T-cell therapies that differ in the costimulatory domain demonstrate efficacy in early-phase trials[97,98]. In 2021, the FDA approved idecabtagene vicleucel (ide-cel, Bristol-Myer Squibb), a BCMA-directed genetically modified autologous T cell therapy, for MM patients that had not responded to > 4 different prior treatments[99]. In 2022, ciltacabtagene autoleucel (cilta-cel), a CAR T-cells with 2 BCMA-targeting single-domain antibodies, was evaluated in RRMM patients with poor prognosis[100]. A single infusion of cilta-cel yielded early, deep, and sustained responses in heavily pretreated patients leading to regulatory approval. CAR T cell therapies have limitations that include life-threatening toxicities, modest antitumor activity, antigen escape, restricted trafficking, and limited tumor infiltration[101-103]. The ECM is composed of fibrous glycosaminoglycans and proteoglycans that act as a physical barrier to CAR T cells and prevent their penetration and infiltration of tumors. Matrix-degrading agents that improve immune cell infiltration may enhance the efficacy of CAR-T cells[104-108].

Bispecific T cell engagers (BiTEs) are novel antibody constructs targeting T cells to a tumor antigen. The prototypical BiTE- blinatumumab (Glaxo-Smith Kline) targets CD3 and CD19 to facilitate T cell-mediated killing of relapsed acute lymphocytic leukemia (ALL) cells [Table 1][109,110]. BiTEs may promote downregulation of their target antigen as a mechanism of immune escape, as evidenced by a metanalysis of ALL patients initially treated with blinatumomab exhibiting increased relapse after CAR T therapy and decreased event-free survival with a trend towards exhibiting more CD19 dim disease. Thus, the development of BiTEs and CAR-T cells with differing target ligands is of clinical interest. Multiple BiTEs have shown promising results in MM including several anti-BCMA/CD3 conjugates as well as talquetamab (Janssen Pharmaceutical Companies of Johnson & Johnson), an anti-GPRC5DxCD3 conjugate that targets endogenous T cells to MM cells with a less severe side effect profile than CAR-T cells with step-up outpatient dosing that can be given to the transplant-ineligible patients[111-113]. The immunosuppressive nature of BMSCs poses a significant hurdle to anti-myeloma immunotherapies. Recently, it was shown that MM or AML cell co-culture with the stromal cell lines HS-5 and HS-27a protected the tumor cells from bispecific antibodies that target CD123 and BCMA[114]. The reduction in T cell effector responses was correlated with impaired CD3 redirection cytotoxicity. Cell-cell contact of tumor cells with stromal cells was thought to decrease T cell activation. Agents that inhibit the very late antigen 4 (VLA4) adhesion pathway may be combined with CD3 redirection to reduce stroma-mediated inhibition of T cell activation. The results lend support to inhibiting VLA4 functional activity as well as administering CD3 redirection therapeutics as a combinatorial regimen that enhances antitumor immunity.

CONCLUSIONS AND FUTURE PERSPECTIVES

The introduction of IMiDs demonstrated the clinical value of immunotherapeutic approaches for the treatment of MM. However, BM-mediated therapeutic resistance promotes tumor escape and immune evasion that represent obstacles to extending patient outcomes. Recently developed myeloma-directed immunotherapies, e.g., monoclonal antibodies, CAR-T cells, antibody-drug conjugates (ADCs), and BiTEs represent the emerging phase of myeloma care[115-118]. Similar to the mechanisms of resistance observed following the administration of cytotoxic chemotherapy, PIs and IMiDs, novel strategies are needed to prevent or overcome resistance to immunotherapies. Numerous immune cell types, e.g., Tregs, Bregs, MDSCs, macrophages, dysfunctional DCs, MSCs, osteoclasts, as well as the ECM, modulate and suppress T- and NK cell activity. BM-mediated immune exhaustion as well as immune checkpoint proteins on T- and NK cells and their corresponding ligands on MM cells, e.g., PD1/PDL-1, or T cell immunoglobulin and tyrosine-based inhibitory motif (TIGIT) domains, represent additional obstacles[119-121]. Innovative platforms will provide the foundation for the next paradigm shift in myeloma to overcome current limitations and improve high-risk and newly diagnosed patient survival[115-117].

DECLARATIONS

Authors' contributions

Conceived the article, prepared the figure and table, and wrote the manuscript: Ignatz-Hoover JJ, Driscoll JJ
Made a substantial, direct and intellectual contribution to the work, and approved the final manuscript for publication: Ignatz-Hoover JJ, Driscoll JJ

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Case Report

Open Access

A patient with relapsed high-grade serous ovarian carcinoma with somatic *RAD51C* mutations treated with PARPi monotherapy: a case report

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Abstract

We report our experience in the management of a relapsed ovarian cancer patient with somatic *RAD51C* mutation, treated with olaparib monotherapy. The patient was diagnosed with stage 4 high-grade serous ovarian carcinoma and was treated with neoadjuvant chemotherapy, cytoreductive surgery, and postoperative chemotherapy. After a second cancer recurrence, she underwent FoundationOne CDx testing following disease progression on multiple lines of chemotherapy. Based on the FoundationOne CDx results, olaparib monotherapy was started. After 13 months of therapy, all lesions responded to the treatment, and she achieved complete response as demonstrated by normalization of the levels of CA125 and positron emission tomography-computed tomography (PET-CT). We plan to continue olaparib monotherapy until disease progression.

Keywords: Ovarian cancer, PARP inhibitor, *BRCA*, *RAD51C*

INTRODUCTION

Recommended treatment for patients with relapsed ovarian cancer depends on platinum-free intervals. Generally, patients who have recurrence after 6 months of completion of platinum-based chemotherapy are

considered platinum-sensitive, while those who recur within six months are considered platinum-resistant. Patients with platinum-sensitive relapse are typically treated with platinum-based regimens, while those with platinum-resistant disease are often given second-line single-agent chemotherapy such as gemcitabine, pegylated liposomal doxorubicin, weekly paclitaxel, or topotecan. It is well recognized that the time to progression usually shortens with subsequent relapses, so that patients encounter shorter treatment-free intervals. Following consecutive therapies, the median duration of progression-free survival often leaves patients with limited or no treatment-free interval and accumulating toxicities.

BRCA1 and *BRCA2* genes are the most identified germline mutations in patients with ovarian cancer, and their proteins are important for DNA repair. Mutations in these genes lead to homologous recombination (HR) deficiency, which may be targeted in the treatment of ovarian cancer with poly ADP-ribose polymerase inhibitors (PARPi)[1]. Among other HR-related genes which have been found to significantly increased the ovarian cancer risk are *RAD51C* and *RAD51D*, coding for proteins that support the process of DNA repair[2]. In epithelial ovarian cancers, *RAD51C* and *RAD51D* mutations were found in 0.41%-0.68% and 0.35%-1.13% of patients, respectively[2-4]. Tumors with mutations in *RAD51C* and *RAD51D* have been found to be *BRCA*-like with high genomic loss of heterozygosity and responded to PARPi (rucaparib) at similar rates to *BRCA*-mutated disease[5].

PARPi are a type of targeted cancer treatment that block the protein PARP, an enzyme that assists in the repair of damaged DNA. In cancer treatment, blocking PARP may help prevent cancer cells from repairing their damaged DNA, leading to cell death[5]. Several PARPi have been approved by the US Food and Drug Administration (FDA) for the treatment of ovarian cancer including olaparib, niraparib, and rucaparib.

We report our experience in the management of a relapsed ovarian cancer patient with somatic *RAD51C* mutation, treated with olaparib monotherapy.

CASE REPORT

History, presentation, and initial treatment

A 74-year-old woman first presented in October 2016 with abdominal pain and distension. She had a history of breast cancer treated with lumpectomy and adjuvant radiotherapy in 2009 and autoimmune hypothyroidism. Pelvic ultrasound showed a 7 cm mass in the Pouch of Douglas and gross ascites. Tumor marker evaluations showed elevated levels of cancer antigen (CA) 125 (1762 U/mL). Paracentesis was performed and peritoneal fluid cytology showed metastatic adenocarcinoma with immunohistochemical markers favoring female genital tract origin (CK7 and PAX8 positive, CK20 negative). Subsequent positron emission tomography-computed tomography (PET-CT) scan showed extensive hypermetabolic lesions in the peritoneum and bilateral adnexa with metastasis to lymph nodes, liver, and spleen, consistent with stage 4 ovarian cancer. After four cycles of neoadjuvant carboplatin and paclitaxel, CA125 decreased to 60 U/mL. PET-CT showed good partial response: previous peritoneal, nodal, liver, and spleen metastases had all resolved, and there was a reduction in the size of the adnexal mass. She then underwent interval debulking surgery including total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy in January 2017. There was some miliary disease left on the mesentery after the debulking surgery. Histology showed high-grade serous carcinoma involving both ovaries, omentum, and peritoneum. The patient received 6 cycles of post-surgical chemotherapy comprising cisplatin and paclitaxel until July 2017. Carboplatin was substituted with cisplatin due to thrombocytopenia. PET-CT performed upon completion of chemotherapy showed complete response and CA125 also normalized (11.1 U/mL). Germline *BRCA1/2* mutation test was negative.

Chemotherapy for recurrence

The patient had the first disease relapse seven months later, in February 2018. PET-CT showed recurrence in the peritoneum, liver, and lymph nodes. She was treated with 6 cycles of cisplatin, gemcitabine, and bevacizumab, due to which she had recurrent grade 4 thrombocytopenia and grade 3 anemia requiring platelet and blood transfusion during the course of the chemotherapy. Following 6 cycles of chemotherapy, PET-CT in September 2018 showed a complete response. She then received 11 more cycles of maintenance bevacizumab until May 2019. In June 2019 (nine months after completion of the last chemotherapy), she had a second relapse with nodal lesions in the abdomen and left supraclavicular fossa. In view of poor marrow reserve, advanced age, and previous chemotherapy, she was prescribed pegylated liposomal doxorubicin instead of platinum-based chemotherapy. Subsequently, the disease progressed despite multiple lines of chemotherapy including four cycles of pegylated liposomal doxorubicin, 6 cycles of topotecan, and five cycles of docetaxel until September 2020. PET-CT in October 2020 showed hypermetabolic lymphadenopathies in the abdomen, thorax, and left supraclavicular regions and liver metastasis.

PARPi monotherapy

She then underwent FoundationOne CDx™ (Foundation Medicine, Inc) testing, which found *RAD51C* mutations in the tumor. Testing for germline *RAD51C* had not been done due to limited availability for testing in our setting. The patient was started on 300 mg olaparib monotherapy twice daily in October 2020. The CA125 just before the commencement of olaparib was 685 U/mL. Due to grade 3 anemia (Hb 7.9 g/dL), the dose of olaparib was reduced to 200 mg twice a day in February 2021. The levels of CA125 gradually decreased and normalized, with the latest results of 9 U/mL [Figure 1]. PET-CT in November 2021 showed a complete response. Last seen in December 2021, the patient was well and continued olaparib therapy.

DISCUSSION

Recently, PARPi have significantly changed the landscape of treatment for advanced and relapsed ovarian cancer. Olaparib, a PARPi, has demonstrated antitumor activity among patients with relapsed ovarian cancer who have a *BRCA1/2* mutation[6,7]. Olaparib monotherapy is approved by the US Food and FDA for the treatment of patients with relapsed ovarian cancer who have a germline *BRCA* mutation and have received three or more prior lines of chemotherapy. In pooled data from 6 trials, the overall response rate of olaparib was 31% in this group of patients, with responses seen in both platinum-sensitive and platinum-resistant diseases[8]. In a phase II trial (ARIEL2, a single-arm, open-label study of the PARPi rucaparib in relapsed high-grade ovarian carcinoma), *RAD51C* and *RAD51D* mutations were associated with the clinically meaningful activity of rucaparib similar to that of *BRCA* mutations. Of 206 patients, there were four patients with *RAD51C* mutations, of whom three patients had a partial response and one patient had stable disease with rucaparib therapy[5,9]. Furthermore, a case report on a heavily pretreated patient with ovarian carcinosarcoma harboring a germline *RAD51D* mutation demonstrated excellent and durable partial response with olaparib monotherapy[10]. Extrapolated from this information and the suggestion from the FoundationOne CDx report of our patient, she was given olaparib monotherapy after she failed to respond to multiple lines of chemotherapy. Olaparib was used on our patient as rucaparib was not available locally. At the time of writing, our patient has been on olaparib monotherapy for 14 months and tolerated it well. To our surprise, all lesions responded to the treatment, and she achieved complete response after 13 months of therapy, as demonstrated by the normalization of the levels of CA125 and PET-CT. We plan to continue olaparib monotherapy for the foreseeable future until disease progression.

Recently, several guidelines, including the US National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO), and European Society of Gynaecological Oncology (ESGO), recommend *BRCA1/2* mutations testing upon confirmation of ovarian, fallopian tube, or primary

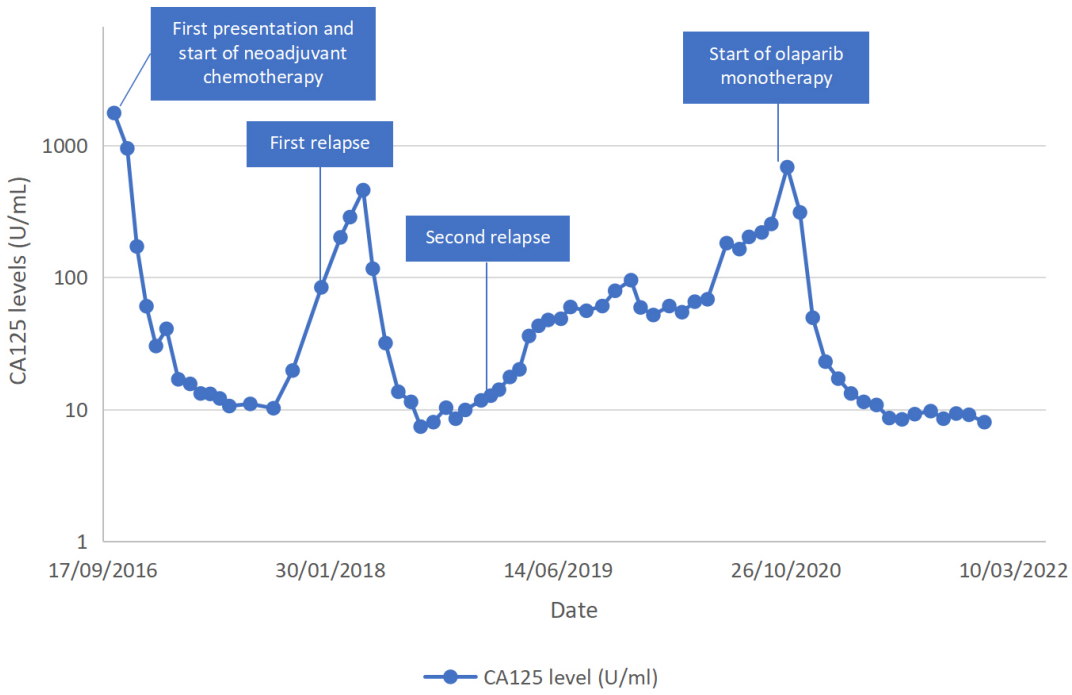

Figure 1. The blood levels of tumor marker CA125 of the patient.

peritoneal cancer, the results of which could be used for maintenance therapy selection following first-line treatment[11,12]. With mutation testing earlier in the course of the disease nowadays, hopefully, the treatment for subsequent relapse can be improved. Although our patient did not have germline *BRCA1/2* mutations, she benefitted from the tumor mutation testing, which allowed for precision medicine, an innovative approach to tailoring cancer treatment to specific information of individuals, such as genetic makeup or genetic profile of the tumor. Our patient underwent FoundationOne CDx test, which is an FDA-approved comprehensive genomic profiling to identify patients who may benefit from specific FDA-approved targeted therapies. The test was performed following the failure of multiple lines of chemotherapy for second disease relapse. Retrospectively, if she had the somatic mutations testing earlier in the course of the disease, she could have benefitted from PARPi therapy sooner, without needing to undergo ineffective chemotherapy regimens and their toxicity. Therefore, patients who have disease relapse should be counseled for somatic and germline mutation testing sooner to allow for personalized targeted therapy.

Following treatment with PARPi, a key question is whether our patient should be rechallenged with platinum-based chemotherapy and would she respond to the treatment in the subsequent disease relapse. Germline or somatic mutations in HR genes are found in around one-third of patients with epithelial ovarian cancer[3]. One study that investigated somatic and germline mutations in 13 HR genes including *RAD51C* demonstrated that somatic mutations in other HR genes have similar survival outcomes and response rates to platinum-based chemotherapy as germline *BRCA1/2* mutations[3]. Theoretically, platinum-based chemotherapy could be considered for our patient in subsequent disease relapse. However, it is unclear whether she could tolerate the toxicities of the treatment in view of poor marrow reserve previously.

In conclusion, our case highlights the potential use of olaparib monotherapy in patients with relapsed ovarian cancer with somatic *RAD51C* mutations when another cytotoxic therapy has failed, as well as the importance of genomic testing to allow for personalized targeted cancer therapy.

DECLARATIONS

Authors' Contribution

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Ngu SF, Ngan HYS, Chan KKL

Performed data acquisition, as well as provided administrative, technical, and material support: Ngu SF

Availability of data and materials

Not applicable.

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None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

A written informed consent for publication has been obtained from the patient.

Consent for publication

A written informed consent for publication has been obtained from the patient.

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Cancer Drug Resistance

Review

Open Access

Drug resistance in metastatic castration-resistant prostate cancer: an update on the status quo

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Abstract

Prostate cancer (PCa) is a leading cause of cancer-related morbidity and mortality in men globally. Despite improvements in the diagnosis and treatment of PCa, a significant proportion of patients with high-risk localized disease and all patients with advanced disease at diagnosis will experience progression to metastatic castration-resistant prostate cancer (mCRPC). Multiple drugs are now approved as the standard of care treatments for patients with mCRPC that have been shown to prolong survival. Although the majority of patients will respond initially, primary and secondary resistance to these therapies make mCRPC an incurable disease. Several molecular mechanisms underlie the development of mCRPC, with the androgen receptor (AR) axis being the main driver as well as the key drug target. Understanding resistance mechanisms is crucial for discovering novel therapeutic strategies to delay or reverse the progression of the disease. In this review, we address the diverse mechanisms of drug resistance in mCRPC. In addition, we shed light on emerging targeted therapies currently being tested in clinical trials with promising potential to overcome mCRPC-drug resistance.

Keywords: Prostate cancer, mCRPC, androgen receptor, drug resistance, novel targeted therapeutics

INTRODUCTION

Prostate cancer (PCa) is a leading cause of mortality and morbidity among men worldwide, where 12.5% of men are prone to be diagnosed with PCa during their lifetime[1]. According to the Globocan data collected from 185 countries in 2020, an estimated 1.4 million new PCa cases were diagnosed, with an incidence rate of 37.5 per 100,000 males, accounting for 14.1% of all male cancer diagnoses. In addition, 375,000 deaths due to PCa were recorded, comprising 6.8% of cancer deaths in men[2]. PCa was discovered to be androgen-sensitive by Huggins and Hodges in 1941[3]. The activation of androgen receptor (AR)-mediated signaling is an essential route for regulating PCa cell growth and proliferation; thereby, alterations in this cascade hold a great influence on cancer progression[4]. This relation between androgen and PCa leads to androgen deprivation treatment (ADT), which includes surgical castration through orchiectomy to remove the source of androgen or medical castration through antiandrogen and gonadotropin-releasing hormone analogs, thus reducing androgens to castrate levels[3]. Interventions such as prostatectomy or radiotherapy along with ADT for localized PCa are the standard of care for localized disease, yet ADT remains the backbone of first-line treatment for locally advanced and metastatic PCa[5]. For patients treated with long-term gonadal androgen suppression, PCa cells become resistant to ADT within 2-3 years and the malignancy progresses even when the serum testosterone level is below the castrate level. This stage of prostate cancer, known as castration-resistant prostate cancer (CRPC), can progress to the lethal form of metastatic CRPC (mCRPC), resulting in rapid mortality with mean overall survival historically of 16-18 months[6].

Although curative treatment has not yet been reached, multiple therapeutic modalities for mCRPC patients have been accomplished[7-16] [Table 1].

Inhibitors of CYP17

The emerging speculative conveyance regarding the defiance of castration resistance classes to androgen manipulating therapies has been fully debunked recently, thus reemphasizing the chief role of the androgen signaling axis as a subject to therapy development[17]. Cytochrome P450 enzyme 17 (CYP17) has been recognized as a vital mediator in steroidal biosynthesis, especially androgen. To impede this vicious pathway, several drugs, such as antifungal ketoconazole, were depicted as potential options; however, prohibitive toxicities paired with minute survival benefits raised the need for better alternatives[18]. Abiraterone (ABI) acetate functions as an irreversible selective steroidal agent for the microsomal complex CYP17, thus restraining two functionalities: cortisol biosynthesis in the adrenal gland (via C21 17-α-hydroxylation) and steroid production in testis and adrenal gland (lyase property)[19]. As a result, both testosterone supply and cortisol will be inhibited, causing a flux of augmented steroid levels upstream of the target halt. Therefore, prednisone (PDN), a corticosteroid that binds to the glucocorticoid receptor (GR), is often co-administered with ABI, seeking in return a diminished side effect for mineralocorticoid excess syndrome[20]. Significantly ameliorated radiographic progression-free survival (rPFS) and overall survival were reported in a large Phase III trial for asymptomatic CRPC patients treated with ABI (Abiraterone) and PDN compared to PDN alone (16.5 *vs.* 8.2 months; HR: 0.52; 95%CI: 0.45-0.61; $P < 0.0001$)[9]. Based on the data presented by such trials, the FDA approved ABI as a management therapy for CRPC patients. Subsequently, the drug has also been shown to improve survival in patients with hormone-sensitive metastatic disease and is also approved as first-line therapy in combination with ADT[21].

AR antagonists

The oral AR antagonist enzalutamide (ENZ) lashes with high affinity, impeding nuclear receptor

Table 1. Summary table of the drugs and agents indicated for mCRPC

Class	Agent	MOA	Studies enrolled with indications	Trial result
Hormonal therapy	Abiraterone Acetate	Irreversible selective inhibitor of CYP17-alpha-hydroxylase and C17, 20-lyase coupled with a modest AR antagonist activity[7]	COU-AA-301; post-docetaxel[8]	ABI increased median overall survival when compared to the placebo group by 3.9 months
			COU-AA-302; pre-docetaxel[9]	ABI significantly increased median overall survival when compared to the placebo group by 8.2 months
AR antagonists	Enzalutamide	AR antagonist impedes nuclear receptor translocation and DNA binding, induces apoptosis	AFFIRM; post-docetaxel[10]	Overall survival increased by 4.8 months in comparison to the control group
	Darolutamide		ARCADES[11]	86% of patients treated with 1400 mg dose of darolutamide had a 50% or greater decrease in PSA
Cytotoxic chemotherapy	Docetaxel	Inhibits microtubular depolymerization arresting their function	TAX 327; first-line chemotherapy[12]	DOC combined with prednisone increased overall survival by 2.9 months when compared to the control group treated with mitoxantrone plus PDN
	Cabazitaxel		TROPIC, Phase III, randomized, open-label[13]	Overall survival increased by 2.4 months in the treatment group (CBZ+ PDN) compared to patients treated with a combination of PDN and mitoxantrone
Calcium-mimetic	Ra-223	Emits high energy alpha particles after adhering selectively to regions of elevated bone turnover	ALSYMPCA, Phase III, randomized[14]	Patients placed on Ra-223 treatment had 3.6 months increase in survival compared to the placebo group
PARP inhibitor	Olaparib	Impede PARP causing cumulative DNA and cell damage	PROfound Phase III, randomized[15]	Patients with mutations in *BRCA1*, *BRCA2*, or *ATM* genes assigned to receive olaparib experienced a 4.4-month increase in survival compared to the control arm receiving ENZ or ABI plus prednisone
Dendritic-cell vaccine	Sipuleucel-T	Infused within APCs to present PAP peptides *in vivo* activating CD4+ and CD8+ T cells	IMPACT trial, Phase III[16]	Utilizing sipuleucel-T extended overall survival among men with mCRPC by 10 months

MOA: Mode of action; AR: androgen receptor; CYP17: Cytochrome P450 enzyme 17; PSA: prostate-specific antigen; ABI: abiraterone; DOC: docetaxel; PDN: prednisone; CBZ: cabazitaxel; ENZ: enzalutamide; PARP: poly(ADP-ribose) polymerase; APC: antigen-presenting cells; PAP: prostatic acid phosphatase.

translocation and DNA binding, thereby inducing apoptosis[22]. Based on the results of two Phase III placebo-controlled studies where a 4.8-month median overall survival benefit was reported in chemotherapy-naïve mCRPC patients, both the FDA and EMA ratified ENZ usage for mCRPC previously treated with docetaxel[23]. Notably, the steroidal biosynthesis impact was nullified with ENZ utilization, thus shifting away from PDN prescription during the treatment regimen[24].

Cytotoxic chemotherapy

By reversibly stabilizing the microtubules and promoting their assembly, docetaxel (DOC) fosters a mitotic block on cancerous cells. After augmentation of these bundles, the natural dynamics of mitosis would be impaired, consequently leading to apoptosis, which is further induced by phosphorylation of the Bcl-2 oncoprotein[25]. Furthermore, impaired microtubules functionality surges p53 buildup in the nucleus, thus driving its downstream signaling forward[26]. Notably, due to their impact on microtubules, it has been shown that taxanes also possess antiangiogenic capabilities paired with impeding AR trafficking and

accumulation[27]. This drug was granted FDA and EMA approval based on the results of the TAX 327 study conducted on patients diagnosed with CRPC and subjected to DOC treatment paired with PDN compared to the standard approach of mitoxantrone and PDN in the control arm. The results demonstrate an overall increased survival benefit (19.2 months *vs.* 16.3 months; $P < 0.004$) reinforced with significant amelioration in pain relief[27]. The incorporation of PDN augments the efficacy of DOC and results in superior outcomes as a result of the additive benefit from the two drugs, each known to be effective in PCa through targeting different signaling, AR and GR. In addition, prednisone also has anti-inflammatory properties and can treat pain, nausea, and edema[28-30]. Moreover, the cytotoxic tubulin-binding drug cabazitaxel (CBZ) has demonstrated a prevailing advantage in enhancing the survival of patients with mCRPC after being assigned to a DOC-based treating regimen, causing a 30% decline in risk of death when compared to those treated with mitoxantrone (panel). These results granted CBZ US Food and Drug Administration approval for being prescribed as a second-line treatment in mCRPC[13].

DRUG RESISTANCE IN MCRPC

Despite the significant survival benefit of the currently approved therapies, which can alleviate symptoms and prolong overall survival, mCRPC remains incurable as primary and secondary resistance develops rapidly[31]. Drug resistance can develop due to mechanisms intrinsic to the biology of PCa or by more general mechanisms shared with diverse tumor types, as can be seen in Figure 1.

AR amplification and overexpression

AR gene amplification has been recognized in clinical studies as the most common genetic alteration deriving AR reactivation and progression to CRPC[32]. AR overexpression can result from different mechanisms such as gene amplification, increased histone acetylation/phosphorylation at enhancers sites, overexpression of co-regulators, or enhanced protein stability[33,34]. The increase in AR expression was found to be consistently linked with the development of resistance to ADT, resulting in PCa progression from a castration-sensitive phenotype to a castration-resistant one. This increase is capable of sensitizing PCa cells to castrate concentrations of androgen and converting the action of AR antagonists to agonists[24,35].

Among patients with CRPC, up to 80% exhibit a significant upregulation in AR transcripts and protein levels[36], in comparison to < 1% of the primary androgen-dependent PCa cases[37]. This aberration has been detected, at high frequency, in circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) and is more common in pretreated CRPC patients than in treatment-naïve patients. A study using liquid biopsies and ctDNA showed that 50% of CRPC patients, pretreated with either ENZ or orteronel (a CYP17A1 inhibitor), evidenced AR amplification[38,39]. One *in vivo* study utilizing CRPC xenografts treated with ABI showed a three-fold increase in AR expression[40]. In a separate *in vitro* study, a bicalutamide-resistant LNCaP cell line was shown to display an overexpressed AR and hyper-sensitivity to minimal levels of androgen[41]. *In vitro* studies using ENZ-resistant LNCaP cells showed an increase in AR levels compared to naïve LNCaP cells[42].

AR point mutations

AR point mutations are also more frequent in CRPC than in primary androgen-sensitive PCa, especially among patients pretreated with ADT. Several studies evidenced somatic AR point mutations in ~10% of CRPC tissues, whereas none were found in any of the primary PCa tissues examined[36,37,43]. This is consistent with results obtained in a previous study that performed a targeted AR sequencing in a cohort of 181 primary cancers and 37 CRPCs. The study revealed that somatic AR point mutations were found to occur only in CRPC and more frequently in patients subjected to prior antiandrogen treatments[44]. Supposedly, when AR signaling is more effectively suppressed, clonal selection of tumor cells will enhance AR somatic

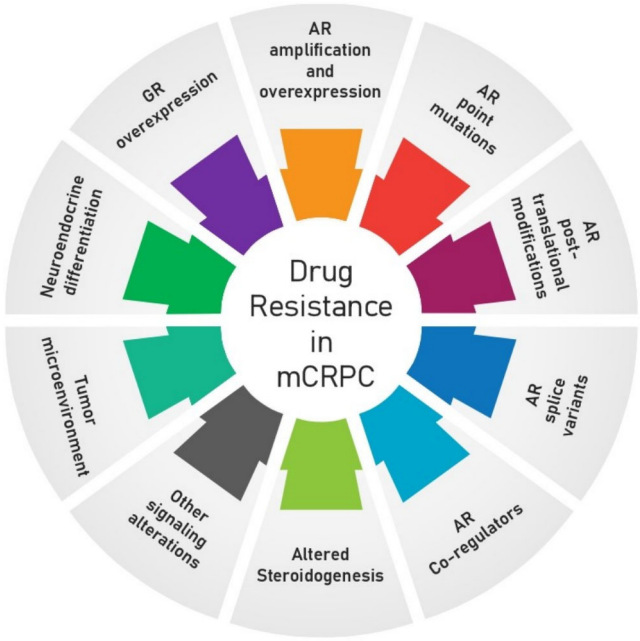

Figure 1. Mechanisms of drug resistance in mCRPC. Several mechanisms of drug resistance are well defined in CRPC, including AR amplification and overexpression, AR point mutations, AR post-translational modifications, AR splice variants, AR co-regulators, altered steroidogenesis, GR overexpression, neuroendocrine differentiation, tumor microenvironment, and other signaling alterations. AR: Androgen receptor; GR: glucocorticoid receptor; mCRPC: metastatic castration-resistance prostate cancer.

mutations, subsequently yielding aberrant transcriptomes[43]. There are > 100 clinically-relevant somatic point mutations detected in AR[44]; the majority are single-base substitutions and are clustered in the ligand-binding domain (LBD). The modifications in LBD alter the steroid-binding pocket and result in broadening ligand specificity and AR activation by alternative non-androgen ligands including progesterone, estrogen, and some AR antagonists[45].

Four main recurrent LBD point mutations are described in several studies: (1) L702H, a leucine to histidine substitution at amino acid 702; (2) W742C, a tryptophan to cysteine substitution at amino acid 742; (3) H875Y, a histidine to tyrosine substitution at amino acid 875; and (4) T878A, a threonine to alanine substitution at amino acid 878 (T878A). These mutations are present in approximately 15%-20% of CRPC cases[46,47]. The gain of function mutation "T878A" described in the LNCaP cell line was shown to confer an expansion of binding specificity to AR by both steroid hormones (e.g., progesterone) and first-generation antiandrogens (e.g., bicalutamide or flutamide)[48,49]. L702H, a mutation that causes glucocorticoid-mediated AR activation, is associated with primary resistance to ABI[50]. In another study, patients harboring T878A and L702H mutations showed poor prostate-specific antigen (PSA) response after ABI or ENZ treatment[38]. In addition, several AR mutations were shown to be capable of converting the AR antagonists into agonists, as seen with F877L and F876L mutations associated with resistance to second-generation antiandrogens ENZ and ARN-509[50,51]. In line with other studies, the data obtained validate the significance of AR mutations in deriving drug resistance.

AR post-translational modifications

AR post-translational modifications (PTMs), including serine/threonine and tyrosine phosphorylation, acetylation, methylation, ubiquitination, and sumoylation, have essential roles in enhancing AR activity through maintaining protein stability, nuclear localization, and transcriptional activity[52,53]. Thereby, PTMs may contribute to AR reactivation and acquisition of drug resistance in mCRPC[54]. A study indicated that

the usage of hypomethylating drugs has the effect of reversing and delaying progression to mCRPC. One potential underlying mechanism is through downregulating DNA methyltransferase 1-dependent STAT3 activity[55]. Phosphorylation of AR residues plays a significant role in activating AR and promoting continued PCa cell growth. The treatment of the castrate-recurrent CWR-R1 cell line with EGF under low androgen conditions was shown to promote AR transcriptional activity through the phosphorylation of AR at serine 515 and serine 578, MAPK, and PKC consensus sites, respectively[56]. A prostate tissue microarray analysis indicated the intracellular non-receptor tyrosine kinase (NRTK) Ack1 to induce AR phosphorylation at Tyrosine 284 and correlate positively with disease progression and negatively with the survival of PCa patients. In activated Ack1-expressing prostate cells, treatment with antiandrogens failed to affect the expression and activation of AR[57]. In addition, the NTRK SRC proved clinically to be at high levels in mCRPC and to mediate AR tyrosine phosphorylation and subsequent pathway activation[58-61].

Ubiquitination is another PTM that was shown to have an important role in regulating AR activity. RNF6, a ubiquitin ligase with an AR enhancing role, acts by inducing AR ubiquitination and promoting AR transcriptional activity. RNF6 was detected t at elevated levels in hormone-refractory human PCa tissues[62]. Another study identified ubiquitin-specific protease 12 (Usp12) as a positive regulator of AR. Usp12, in complex with the cofactors Uaf-1 and WDR20, interacts with and deubiquitinates AR, resulting in increased protein stability and transcriptional activity of AR[63].

AR co-regulators: co-activators, co-repressors, and chromatin remodelers

A series of co-regulator protein factors such as co-activators, co-repressors, and chromatin remodelers are known to regulate AR transcriptional activity by being co-recruited to chromatin and binding directly or in a protein complex to AR to regulate gene activity. The balance of co-activator and co-repressors is required for the proper regulation of AR-mediated transcription. Misbalance in these factors leads to higher AR activity and less active antagonism by antiandrogens contributing to drug resistance and mCRPC progression[64,65]. FKBP51, a co-activator and a target gene of AR, was detected at upregulated levels in relapsed LAPC-4 tumors grown in castrated mice[66]. FKBP51 improved the formation of a super chaperone complex via recruiting p23 co-chaperone to ATP-bound Hsp90, which in line keeps AR with a high-affinity conformation for ligand-binding, consequently promoting androgen-mediated AR transcriptional activity and growth[66,67].

Elevation of AR signaling can also occur through the loss of AR regulatory repressing signals. For example, retinoblastoma (Rb), a well-known tumor suppressor, was shown to be highly reduced in mCRPC and to be associated with tumor recurrence. The loss or inactivation of Rb induced an E2F1-mediated increase in the levels of AR mRNA/protein and two relevant target genes, *PSA* and *TMPRSS2*, consequently promoting castrate-resistant growth and resistance to bicalutamide in PCa cells[68].

A recent study identified and verified myosin heavy chain 9 (MYH9) as a novel AR cofactor. The data show that inhibiting MYH9 in an androgen-independent cell line (LNCaP-AI) promoted AR nuclear translocation and enhanced the expression of PSA, indicative of the MYH9 role as a co-repressor to nuclear AR signaling and a novel factor mediating the progression of CRPC[69].

Other pioneering factors, i.e., FOXA1 and GATA2, act as chromatin remodelers at enhancer sites to regulate the expression and activity of AR in mCRPC. FOXA1 induces open chromatin conformation to allow the binding of other transcription factors, aiding in an enhanced AR transactivation and mCRPC progression[70]. CTCs isolated from patients with ABI- or ENZ-resistant metastasis showed amplification in the FOXA1 gene in > 30% of CRPC patients, pinpointing the crucial role of FOXA1 in AR regulation and

tumor progression[71]. In addition, GATA2 was shown to boost AR activity and CRPC progression. Overexpression of GATA2 was correlated with more aggressiveness in PCa[72]. High expression of GATA2 in CRPC cell line "ARCaPM" brings about an increased IGF-2 expression and consequently confers chemotherapy resistance[73]. An additional important AR co-regulator and chromatin remodeling factor is homeobox B13 (HOXB13), which acts by interacting with AR and binding to its target loci[74]. Analysis of transcriptome expression from several databases revealed that the expression of HOXB13 is elevated during the progression of the primary PCa to CRPC[75]. In a separate study, HOXB13 was shown to be highly expressed in hormone-refractory tumors compared to tumors without PSA after initial treatment[76]. Interestingly, HOXB13 also mediates the oncogenic function of AR splice variants, mainly ARV7, and acts as a pivotal upstream regulator of AR-V7–driven transcriptomes[77]. These findings could explain HOXB13's role in resistance towards antiandrogen treatment.

AR splice variants

Transcriptionally activated AR splice variants (ARVs) are truncated forms of AR that lack the C-terminal LBD region, the binding site for first- and second-generation drugs, hence remain constitutively activated independently of androgen or antagonist action. This ligand-independence feature makes ARVs potentially contributors to disease progression and treatment resistance in mCRPC. ARV1, ARV7, and ARV567 are the most common isoforms, while ARV7 is the most extensively studied one[78,79]. ARV7 is associated with resistance to both ABI and ENZ. Studying ARV7 expression in CTCs revealed its association with clinical resistance to both drugs in men receiving ENZ. Patients with detectable ARV7 levels had poorer PSA response, shorter progression-free survival (PFS), and reduced overall survival (OS) compared to ARV7-negative patients. Similar results were observed in patients treated with ABI. Markedly, the ARV7 level was higher in males pretreated with ENZ and ABI than in treatment-naïve males[80]. The selective siRNA-mediated knockdown of ARV7 in CWR22Rv1 cells inhibited androgen-independent growth and re-established the responsiveness to antiandrogen drugs[81]. In one study, ARV567es was identified and characterized as a contributor to PCa progression and resistance to castration in human PCa xenograft models and a biomarker for patients with early recurrence. In addition, AR-V567es transcripts detected in 23% of CRPC bone metastases were shown to be associated with poor prognosis and short survival[82]. These data strongly support the implication of ARVs as resistance-driving factors in CRPC.

Intra-tumoral and alternative androgen biosynthesis/altered steroidogenesis

Although CRPC develops in the presence of sub-physiological levels of circulating androgens, its progression is associated with the accumulation or synthesis of intra-tumoral androgen and accordingly maintained AR activity[83]. The low plasma levels of androgens following ADT, can be bypassed by the local conversion of adrenal androgens to testosterone and de novo local synthesis of androgens through increased expression of enzymes involved in steroidogenesis, such as CYP17A, in the prostatic tissue. The elevated intra-tumor level of androgens stimulates AR paracrine and autocrine activation, regardless of serum androgen levels[84,85].

The intra-tumor synthesis of steroid hormones was shown to be significantly increased in CRPC patients compared to naïve primary PCa. To compare the levels of androgen, a study utilized autopsies and tissue biopsies from hormone-sensitive and hormone-resistant patient tumors, and the latter was shown to possess high levels of continuous androgen production compared to primary tumors[83]. A primary source of these de novo intra-prostatic androgens are the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione (AD), generated by the action CYP17A1 enzyme. In the prostate, DHEA and AD can act on the AR axis once converted to testosterone and DHT. DHEA also exists predominantly in a sulfated form (DHEA-S). In CRPC, although ABI (CYP17 inhibitor) results in a significant decline of both DHEA and AD, the persistent DHEA-S can serve as a precursor for testosterone and DHT synthesis in prostate

tissue, thus conferring resistance to ABI[86-88]. Studies in CRPC xenografts have shown that several genes involved in the androgen synthesis pathway are upregulated during hormonal therapy. The overexpression of AKR1C3, an enzyme implicated in the steroidogenesis pathway, drives resistance to ABI acetate via increasing androgen synthesis and signaling[89]. In line, downregulating AKR1C3 re-sensitizes resistant cells to ABI treatment. Consistently, AKR1C3 activation has been shown to be associated with ENZ resistance[90]. Other enzymes implicated in steroidogenesis, i.e., SRD5A1 and HSD3B2, were found to be overexpressed in samples from CRPC bone marrow metastases[91].

Glucocorticoid receptor overexpression

The role of glucocorticoids is complex in PCa because of their dual role. Despite that, it is shown that whenever adenosine diphosphate (ADP) is used to antagonize AR signaling, the GR is activated and overexpressed to confer resistance to the treatment used[92]. Both GR and AR have similar structures and mechanisms of action; thus, in mCRPC, whenever a treatment is used to block AR signaling[93], GR is usually activated to attach to nuclear AREs and activate genes that stimulate tumor progression and cell endurance[94]. A preclinical study showed that GR was overexpressed and conferred resistance in *in vitro* cell lines (VCaP and LNCaP/AR) that were treated with apalutamide and ENZ. However, it was shown that VCaP was desensitized to ENZ when GR was knocked out[93]. Another study also showed that CRPC was resistant to DOC when GR was overexpressed. In this study, GR antagonism was used to successfully re-sensitize the cells to DOC[95]. Puhr *et al.*[96] observed that GR is expressed minimally in primary PC tissue and the expression notably increases with long-term treatment with ENZ.

Neuroendocrine differentiation

While only about 1% of all primary PCa are diagnosed as neuroendocrine prostate cancer (NEPC), up to 30% of mCRPCs are NEPC[97]. This profound phenotypic shift, as a result of selection pressure from ADT or potent antiandrogens, from tumors with histological features of adenocarcinoma that express AR to AR-negative neuroendocrine prostate tumors has been termed lineage plasticity[98]. The loss or mutation of both tumor suppressors TP53 and RB1 has been found to be crucial in NEPC differentiation[99]. Tan *et al.*[100] observed Rb protein loss in 90% of NEPC with RB1 allelic loss in 85% of cases. A recent *in vivo* preclinical mouse study found that *Rb1* loss promotes plasticity and metastasis in PCa with TP53, and tumor suppressor phosphatase and tensin homolog *(Pten)* loss causes secondary resistance to therapies that target the AR signal axis[101]. A different preclinical study of *in vitro* and *in vivo* models of human PCa showed evidence of lineage plasticity and a shift from androgen-dependent PCa to androgen-independent NEPC after treatment with ENZ[102]. This phenotypic shift was induced by the loss of RB1 and TP53 and was facilitated through overexpression of the transcription factor SOX2. It was also demonstrated that the inhibition of SOX2 restored the function of TP53 and RB1[102]. At the moment, there are no therapeutics in drug development studies that target the loss or mutations of TP53 or RB1. However, a Phase II study is currently ongoing to evaluate the potential use of alisertib (aurora kinase A inhibitor) in mCRPC and NEPC patients[103].

Tumor microenvironment

The ability of the tumor microenvironment to promote drug resistance is likely linked to the role of the cancer-associated stromal cells in tumor initiation and progression. Tumor microenvironment-derived neuregulin 1 (NRG1), identified in cancer-associated fibroblast (CAF) supernatant, was shown to induce resistance in tumor cells through activation of human epidermal growth factor receptor-3 (HER3) and the subsequent downstream signaling molecules such as MAPK to promote cell proliferation and survival[104]. Blocking the NRG1/HER3 axis was shown to re-sensitize tumors to hormone deprivation *in vitro* and *in vivo*. In addition, patients with mCRPC having increased tumor NRG1 activity showed an inferior response to second-generation antiandrogen therapy[105,106]. Zhang *et al.*[106] showed that ENZ drives in overproduction

of NRG1. It is still unclear how AR inhibitors affect the production of NRG1, but many studies have shown that NRG1 overexpression is correlated with poor outcomes and reaction to ADT.

SPP1, an important extracellular matrix component secreted by multiple kinds of cell types including immune cells, fibroblasts, osteoclasts, smooth muscle, and epithelial cells, is overexpressed in many types of tumors[107,108]. In the GSE32269 dataset, SPP1 expression was shown to be significantly higher in the mCRPC group than in the primary PCa group. The Human Cancer Metastasis Database analysis showed that SPP1 expression levels were significantly higher in bone metastases than in lymph node and posterior peritoneum metastases, thus correlating the expression of SPP1 with the progression of mCRPC[109]. To investigate the relationship between SPP1 expressions and ENZ resistance *in vivo*, Pang *et al.*[108] studied the effect of ENZ treatment on SPP1 knock downed cells (22Rv1 cell line). The results show that SPP1 knockdown significantly inhibited 22Rv1 cell proliferation after ENZ treatment.

Other signaling alterations/implication of growth factors, kinases, cytokines, enzymes...

The aberrant activation of multiple signaling pathways plays a key role in deriving drug resistance in mCRPC. High levels of growth factors including IGF-1, EGF, and TGF-α/β have been reported in mCRPC[110]. A study showed that the overexpression and activation of EGFR mediate DOC resistance in CRPC by inducing AKT-dependent ABCB1 (MDR1) expression[111,112]. Besides, the stimulation of EGFR was shown to derive the activation of Ack1/Tnk2, which is known to correlate positively with the progression to the mCRPC stage[113]. In addition, PCa patients whose tumors showed moderate to strong staining of activated Ack1 displayed a poor prognosis[113].

Several studies assured the association of mCRPC with increased activation of the PI3K-AKT-mTOR pathway[114,115]. Phosphatase and Tensin Homolog (PTEN) has been attributed to the radical amelioration of the PI3K/AKT pathway in nearly 50% of PCa patients. This atypical augmentation of the latter pathway distorts the protein-serine/threonine kinases promoting castration-resistant growth. PI3K-AKT-mTOR pathway interacts and cooperates with several key oncogenic signaling cascades such as MAPK and WNT signaling to facilitate PCa growth and drug resistance[116]. Furthermore, augmented levels of ERKs activation have been reported in recurrent mCRPC patients, and correlative studies have further linked several mutations down the RAS isoforms with the tumorigeneses of PCa[117]. Although collateral activation of the PI3K/AKT and RAS/MAPK pathways have been implicated in several studies, cell proliferation and invasiveness of p63-expressing prostate tumors were solely attributed to the MAPK signaling pathway, which suggests its critical involvement in mCRPC[118].

Cytokines overexpression is also known to trigger tumor progression and drug resistance in CRPC[119,120]. A study reported that inhibiting STAT3, a downstream target of IL-6, results in decreased growth of ENZ-resistant cells. Consistently, blocking STAT3 signaling by inhibiting its phosphorylation was shown to re-sensitize ENZ-resistant cells, hence supporting the implication of the IL-6-STAT3 pathway in ENZ-acquired resistance[121]. Moreover, radically increased IL-6 expression is a direct consequence of the constitutive activation of the NF-κB pathway[122,123]. NF-κB levels influence the expression of an essential PCa biological marker for progression, PSA. Additionally, NF-κB is a key factor in the upregulation of IL-8 cytokine; the latter is involved in the regulation of prostate vasculature and apoptosis[124]. High levels of circulating IL-8 were detected in advanced PCa at a stage when the tumors no longer respond to antiandrogens[125,126]. PCa cells overexpressing IL-8 show reduced effectiveness of bicalutamide[124]. The pro-inflammatory cytokine TNF-α has also been reported to be elevated in CRPC, and such chronic stimulus is a prototypical inducer of NF-κB expression, thereby increasing metastasis, proliferation, and drug resistance[127].

Analysis of whole-exome and transcriptome sequencing of mCRPC biopsies revealed that alterations in DNA-damage repair (DDR) genes including *BRCA2*, *BRCA1*, and *ATM* occur at higher frequencies in mCRPC (19.3%) than in primary PCa, of which 12.7% of the samples were identified with loss of BRCA2[43]. Cells with deleterious mutations in *BRCA1* or *BRCA2* compensate for this loss by increasing their dependency on poly(ADP-ribose) polymerase (PARP) activity for DNA repair[128,129].

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein that is normally expressed in the prostate epithelium. Although it is expressed in other tissues (such as the salivary glands, proximal tubules of the kidney, and small intestine), its levels there are minimal. Importantly, in PCa tissues, PSMA is significantly overexpressed, having the highest expression in advanced PCa and mCRPC. Moreover, following androgen deprivation and hormonal therapy, PSMA expression seems to be increased[130-132].

EMERGING TREATMENTS TARGETING MCRPC

Based on the above, there is a crucial need for novel alternative approaches and drugs that could overcome resistance in advanced PCa stages. In fact, several treatments, which are under development and trials, could emerge as promising therapies for patients with mCRPC and become the next-generation standards of care. Table 2 summarizes the evolving targeted treatments in mCRPC along with their relevant clinical trials[15,133-156].

PARP inhibitors

The PARP superfamily, with at least 18 members, comprises nuclear enzymes involved in the DNA repair machinery of single-stranded breaks (SSBs), cell proliferation, and death[157]. Inhibition of PARP proteins impedes the ligation of SSBs that consequently convert into double-stranded breaks (DSBs). When DDR genes are defective, PARP inhibition leads to the accumulation of DSBs, promoting an enhanced lethal effect known as the "synthetic lethality" that ultimately induces cell death. According to this rationale, PARP inhibitors (PARPi) emerged as a therapeutic approach targeting malignancies with defective DDR genes, particularly BRCA1/2[158-160]. In fact, PARPi application showed success in BRCA-deficient breast and ovarian cancer and promising efficacy in clinical trials involving mCRPC patients[129,161]. Several PARPi have been tested and approved in mCRPC including olaparib, rucaparib, niraparib, talazoparib, and veliparib.

Olaparib (Lynparza) is a small, oral, and bioavailable molecule that selectively binds and inhibits PARP[162]. Mateo *et al.*[134] published two Phase II clinical trials, "TOPARP-A" (NCT01682772) and "TOPARP-B" (NCT01682772), involving patients with mCRPC who had previously received standard treatments and who were treated with olaparib. Both studies demonstrated that patients with defective DDR genes and who were no longer responding to the current treatments exhibited a high response rate to olaparib. Importantly, response to treatment seemed dependent on specific DDR gene defects, as the highest response was achieved in the BRCA1/2 aberrant subgroup[133,134,163].

In May 2020, based on the results of the "PROfound" (NCT02987543) clinical study, olaparib was granted a "breakthrough" FDA approval for the treatment of men with mCRPC harboring germline and/or somatic mutations in DDR genes and who formerly received second-generation antiandrogens[164]. PROfound is a prospective, randomized, Phase III trial. Patients with mCRPC and DDR genes alterations whose disease progressed while receiving ENZ or ABI were randomly assigned to receive either olaparib or ENZ/ABI (control). The results show that patients receiving olaparib had significantly longer median imaging-based PFS than the control group[135]. Moreover, this study was the first to show that patients on opalarib had a significantly longer duration of survival compared to the control[15].

Table 2. Summary of the completed and ongoing clinical trials of emerging therapies in mCRPC

Therapy	Agent(s)	Trial name/Clinicaltrials.gov identifier	Trial phase	Main findings/Comments
PARPi	Olaparib	TOPARP-A/NCT01682772[133]	II	Olaparib improved the RR specifically in patients with DDR gene defects
	Olaparib	TOPARP-B/NCT01682772[134]	II	Olaparib improved the RR specifically in patients with *BRCA1/2* aberrations
	Olaparib or ENZ/ABI	PROfound/NCT02987543[15,135]	III	Olaparib increased PFS and OS
	Rucaparib	TRITON2/NCT02952534[136,137]	II	Rucaparib improved RR and PSA RR in patients with *BRCA* alterations
	Rucaparib or physician's choice of ABI/ENZ/DOC	TRITON3/NCT02975934	III	Ongoing trial
	Niraparib	GALAHAD/NCT02854436[138,139]	II	Ongoing trial; interim results show that niraparib improved RR in patients with *BRCA1/2* biallelic alterations
	Talazoparib	TALAPRO-1/NCT03148795[140]	II	Ongoing trial; interim results show that talazoparib improved RR in patients with *BRCA1/2* alterations
	Talazoparib + ENZ or ENZ	TALAPRO-2/NCT03395197	III	Ongoing trial
PSMA radioligand therapy	LuPSMA	[141,142]	II	LuPSMA showed high RR, low toxicity, and reduction of pain in patients LuPSMA showed a better response than other therapies when rechallenged upon progression
	LuPSMA or cabazitaxel	TheraP/NCT03392428[143]	II	The percentage of patients who achieved PSA_{50} is higher in the LuPSMA group The percentage of patients with AEs is lower in the LuPSMA group
	LuPSMA + best supportive/best standard of care or best supportive/best standard of care	VISION/NCT03511664[144]	III	LuPSMA improved PFS and OS
PSMA-targeted immunotherapy	CAR^+ T cells	NCT01140373[145]	I	3 10^7 CAR^+ T cells/kg was safe with persisting CAR-T cells in peripheral blood for up to two weeks One patient exhibited a long-term response with stable disease status for more than 16 months
	PSMA-targeted/TGFβ-resistant CAR-T cells	NCT03089203[146]	I	Ongoing trial
	Fully humanized anti-PSMA monoclonal IgG1 antibody conjugated to MMAE PSMA-ADC	NCT01695044[147]	II	Discontinued trial as 40% and 31% of the participants showed progressive disease and AEs, respectively
	MEDI3726 (PSMA-ADC linked to pyrrolobenzodiazepine)	NCT02991911[148]	I	AEs occurred in 91% of the patients and 33% discontinued MEDI3726 exhibited responses at higher doses, but treatment was discontinued
	MLN2704 (PSMA-ADC with a humanized monoclonal antibody linked to the maytansinoid DM)	[149]	I/II	MLN2704 had a low PSA_{50} response Neurotoxicity was dose-limiting
	Pasotuxizumab (AMG 212 or BAY 2010112)	NCT01723475[150]	I	AMG 212 had dose-dependent clinical efficacy and manageable safety Relatively long-term response was seen in two patients
	Acapatamab (AMG 160)	NCT03792841[151,152]	I	Acapatamab had an acceptable safety profile, promising PSA_{50} response, stable disease in 8/15 patients, and 14% of the patients continued the treatment for more than 6 months
Androgen receptor degraders	ARV-110	NCT03888612[153]	I	Ongoing trial
PI3K pathway	Ipat (GDC-0068) + ABI or placebo + ABI	NCT01485861[154,155]	Ib/II	Ipat improved PFS and OS

inhibitors				Patients with PTEN loss had prolonged PFS
	Ipat + ABI or placebo + ABI	NCT03072238[156]	III	Ongoing trial; interim results show that Ipat prolonged PFS Two treatment-related deaths occurred in both groups

ABI: Abiraterone; ADC: antibody-drug conjugate; AEs: adverse events; CAR-T cells: chimeric antigen receptor T cells; DDR: DNA-damage repair; DOC: docetaxel; ENZ: enzalutamide; IgG1: immunoglobulin G1; Ipat: ipatasertib; LuPSMA: lutetium-177[^{177}Lu]-PSMA-617; MMAE: monomethyl auristatin E; OS: overall survival; PARPi: poly(ADP-ribose) polymerase inhibitor; PFS: progression-free survival; PSA: prostate-specific antigen; PSA_{50}: > 50% decline in prostate-specific antigen; PSMA: prostate-specific membrane antigen; RR: response rate; TGFβ: transforming growth factor-β.

Recently, Marshall *et al.*[165] performed a retrospective, observational study involving patients with mCRPC having somatic or germline mutations in *BRCA1*, *BRCA2*, or *ATM* who were treated with olaparib. This study aimed to assess whether responses to olaparib are different between men with mutations in *BRCA1/2 vs. ATM*. Indeed, patients with *BRCA1/2* alterations exclusively achieved > 50% decline in prostate-specific antigen (PSA_{50} response). Moreover, these patients had a longer median PFS. Thus, this study demonstrated that men with *BRCA1/2* respond better to opalarib than those harboring *ATM* mutations, and better alternatives should be considered for patients with ATM alterations[165].

These clinical trials helped to establish the efficacy and safety of opalarib as a monotherapy. Other trials investigating opalarib as a monotherapy outside the scope of DDR genes are underway[166]. Moreover, several preclinical and clinical studies testing the synergistic effects of opalarib with other cytotoxic drugs (chemotherapy, AR-directed therapy, immune checkpoint inhibitors, radioligand therapy, radiotherapy, *etc.*) on mCRPC are still ongoing or completed.

Rucaparib is a small, oral, bioavailable PARPi that was evaluated in clinical trials due to its chemosensitization, radiosensitization, and antineoplastic potency[167]. Rucaparib elicits a cytotoxic effect comparable to opalarib[168]. Results of "TRITON2" (NCT02952534) Phase II trial prompted an "accelerated" FDA approval of rucaparib for men with mCRPC and *BRCA* mutations who were previously treated with AR-directed therapy and taxane-based chemotherapy. In this study, rucaparib treatment on patients with *BRCA* alterations showed promising response rates, particularly in comparison to patients with non-*BRCA* DDR gene alteration[136,137,169].

Based on the *ClinicalTrials.gov* website, a Phase III clinical trial, "TRITON3" (NCT02975934), is ongoing. Patients with mCRPC harboring *BRCA1*, *BRCA2,* or *ATM* mutations who progressed on AR-directed therapy but did not receive chemotherapy are actively being recruited. This study aims to assess the patients' response to rucaparib monotherapy *vs.* a treatment of the physician's choice of ABI, ENZ, or DOC to verify the clinical benefit of rucaparib. In addition to TRITON3, other studies are currently active to evaluate the efficacy of rucaparib in combination with nivolumab or chemotherapeutic treatments[160].

Niraparib (Zejula) is a small, oral, once-daily, bioavailable, potent, and highly selective Poly (ADP-ribose) Polymerase Inhibitors(PARPi) with antineoplastic activity[170]. "GALAHAD" (NCT02854436) is an active, ongoing, Phase II clinical trial that aims to assess the efficacy, safety, and pharmacokinetics of niraparib in men with treatment-refractory mCRPC and DNA repair alterations (biallelic alterations). Interim analysis shows that niraparib may have promising efficacy

as a monotherapy for men with mCRPC, specifically those harboring *BRCA1/2* biallelic alteration[138,139]. Niraparib is also being tested in several trials as a combination therapy with other cytotoxic drugs (ABI and radium-223).

Talazoparib (Talzenna) is a small, oral, bioavailable molecule that was demonstrated to be the most potent among PARPi in terms of *in vitro* activity and trapping of PARP on DNA SSBs[171,172]. Between October 2017 and March 2020, participants were recruited and enrolled in "TALAPRO-1" (NCT03148795), an ongoing Phase II clinical trial whereby patients with mCRPC and DDR gene alterations who progressed on standard treatments were eligible and provided with talazoparib. In addition, different clinical trials assessing the efficacy of combining talazoparib with other therapeutics are ongoing including the Phase III TALAPRO-2 (NCT03395197) trial, which compares the combination of talazoparib and ENZ *vs*. ENZ alone[140].

PSMA-based therapies

The overexpression of PSMA, particularly in mCRPC, makes it a potential therapeutic target for PCa. Upon binding to their receptors, PSMA ligands are internalized, leading to persistent retention of the ligand intracellularly. This signaling process seems specific to cancer cells, as in normal cells, a relatively rapid washout takes place. Abusing this feature in cancer cells that have increased PSMA ligand uptake, targeting PSMA by radioligand therapy or by immunotherapy has become an emerging therapeutic approach to treat mCRPC[173,174].

PSMA radioligand therapy

The radioligand targeting PSMA, which has come the farthest in development, is lutetium-177, [^{177}Lu]-PSMA-617 (LuPSMA). It is a small, radiolabeled beta-emitter with a high binding affinity to PSMA. LuPSMA is advantageous by the short-range path length of the emitted beta particle, allowing efficient delivery of the radiation to the target cells while minimizing off-target effects on surrounding tissues[141]. Since 2014, many retrospective and prospective studies have been performed to assess the efficacy and safety of LuPSMA in treating mCRPC.

The German Society of Nuclear Medicine performed the largest multicenter retrospective data analysis that studied the toxicity and efficacy of LuPSMA on 145 mCRPC patients. This study showed promising results for LuPSMA in terms of high efficacy and desirable safety, whereby 40% of the patients responded after a single treatment cycle[175].

Hofman *et al.*[141] performed a Phase II prospective trial involving men with mCRPC who progressed after receiving conventional PCa treatments and had high expression of PSMA. This trial's sample size was expanded, and long-term outcomes were assessed by Violet *et al.*[142]. Analysis of the results of those studies showed a high response rate, low toxicity, reduction of pain in patients with mCRPC who received LuPSMA, and better response over other therapies when rechallenged upon progression.

The results of two recent trials, the "TheraP" (NCT03392428) Phase II prospective study and the "VISION" (NCT03511664) Phase III trial, involving mCRPC patients treated with LuPSMA proved that the latter is a potential novel efficient therapeutic for mCRPC and possible alternative to the approved chemotherapeutic drug CBZ and taxane-based regimens[143,144].

Finally, although LuPSMA is the most studied PSMA radioligand therapeutic agent in recent years, other molecules are also being investigated, such as the alpha-emitting actinium-225 (225Ac-PSMA-617). This is an alpha-emitter with higher potency and a shorter range than beta-emitters[176]. A meta-analysis was

recently published in September 2021 that summarizes the effects of 225Ac-PSMA-617 in mCRPC from nine studies with 263 patients; the authors concluded that 225Ac-PSMA-617 might be a potentially effective therapeutic option for mCRPC patients[177].

PSMA-targeted immunotherapy

In addition to its significantly high expression in mCRPC, PSMA has a large extracellular domain which makes it an ideal target for immune agents[174]. Several approaches for PSMA-targeted immunotherapy exist including chimeric antigen receptor T cells, antibody-drug conjugates, bispecific T-cell engagers, and PSMA-directed vaccines.

a. Chimeric antigen receptor T cells

Chimeric antigen receptor (CAR) is a genetically engineered transgenic T-cell receptor. It consists of an antigen recognition moiety allowing T cells to identify intact specific tumor-associated antigens and T-cell signaling domains that activate T cells. Thus, CAR-T cells combine the properties of antibodies that recognize particular antigens with the cytolytic killing of T cells[178-180].

A Phase I study (NCT01140373) was performed by Slovin *et al.*[145] to assess the safety, tolerability, and efficiency of escalating doses of PSMA-targeted CAR-T cells. Seven patients included in this study received from 10^7 to 3 10^7 CAR-T cells/kg. The highest given dose was shown to be safe with persisting CAR-T cells in peripheral blood for up to two weeks. Importantly, one patient exhibited a long-term response having a stable disease status for more than 16 months[145].

Moreover, a first-time Phase I clinical trial (NCT03089203) to test the safety, feasibility, and efficacy of PSMA-targeted/TGFβ-resistant CAR-T cells is ongoing. The experimental approach strives to overcome the immunosuppressive tumor microenvironment (TME) in mCRPC, to which high levels of TGFβ are a contributing factor. This rationale is based on *in vivo* disseminated PCa models results, where PSMA-redirected CAR-T cells expressing a dominant-negative TGFβ receptor had an increased T-cell proliferation and cytokine release, long-term persistence, and greater tumor elimination[146]. The results of this clinical trial are pending.

b. Antibody-drug conjugate

Antibody-drug conjugate (ADC) technology is an emerging therapeutic approach that is still in its early phases. ADCs are highly specific monoclonal antibodies targeted against specific tumor antigens and chemically conjugated to cytotoxic agents[181]. Target selection is one of the key considerations in ADCs design. The target needs to be highly expressed on the surface of tumor cells *vs.* low to no expression in normal cells. Moreover, the target preferably needs to have internalization characteristics that allow the transport of the antibody to carry the cytotoxic payloads into cancerous cells. These requirements are met in PSMA, making it an attractive target; therefore, several ADCs targeting PSMA in PCa are in clinical development[182].

A Phase II trial (NCT01695044) was performed by Petrylak *et al.*[147], a Phase I trial (NCT02991911) by de Bono *et al.*[148], and a Phase I/II multiple escalating dose trial by Milowsky *et al.*[149] tested different PSMA-ADCs. The different trials showed that, although these drug conjugates had some activity in mCRPC, their effect was accompanied by significant adverse events (such as neutropenia and neuropathy), which led to the discontinuation of some of these treatments. Hence, it was noticed that toxicity related to the long

circulation in the system of PSMA-targeted ADCs limited the success of the clinical trials[183].

c. Bispecific T-cell engagers

Bispecific T-cell engagers (BiTEs) are bispecific antibodies that simultaneously target a T-cell-specific molecule (almost always CD3 chain due to its conserved property) and a TAA, which could be PSMA in the case of PCa[184]. BiTEs showed success in several types of cancer, such as acute lymphoblastic leukemia, where blinatumomab was the first FDA-approved BiTE[185,186].

Hummel *et al.*[150] conducted a first-in-human, dose-escalation, Phase I clinical trial (NCT01723475) to primarily test PSMA CD3 first-generation BiTE pasotuxizumab (known as AMG 212 or BAY 2010112) in patients with mCRPC refractory to standard therapy. In this study, data analysis demonstrated the dose-dependent clinical efficacy and manageable safety of pasotuxizumab in mCRPC, with 3 out of 16 patients exhibiting ≥ 50% PSA response and two patients having a relatively long-term response[150].

Another first-in-human, Phase I study (NCT03792841) to assess acapatamab (AMG 160), a novel next-generation PSMA CD3 BiTE with an extended half-life, in mCRPC was conducted[151]. AMG 160 gave a promising PSA_{50} response and acceptable safety profile in treated patients. This study prompted testing AMG 160 as a combination therapy with other agents (pembrolizumab, ENZ, ABI, AMG 404, and etanercept prophylaxis) in ongoing trials[152,187,188].

d. PSMA-directed vaccines

Cancer vaccines, intended to enhance the immune response against tumor cells by increasing the pool of TAA-specific host T cells, have failed to demonstrate a considerable benefit in PCa thus far (such as the Phase III clinical trials "PROSTVAC" that targets PSA and "GVAX", where both failed to meet their primary endpoint of enhancing the overall survival)[189,190]. However, this approach continues to be investigated, given its potential efficacy, minimal side effects, limited cost, and easy synthesis. Specifically, PSMA is being considered as an appropriate target for the development of PCa vaccines[191]. Using new computational tools to select suitable B-cell epitopes with high antigenicity, the "673RHVIYAPSSHNKYAGE25" peptide was predicted as having the best binding affinity. Future steps involve synthesis of the peptide and testing its *in vivo* efficacy as a potential PSMA-directed vaccine[192].

Androgen receptor degraders

Androgen receptor degraders emerged as an alternative therapeutic strategy to manage mCRPC. One of the tools that allow targeting of AR to degradation is using proteolysis-targeting chimera (PROTAC) novel technology. PROTACs, also known as bivalent chemical protein degraders, are heterobifunctional compounds consisting of two recruiting ligands joined by a linker. One ligand moiety binds to the protein of interest, while the other is specific to E3 ubiquitin ligase (E3). Thus, PROTACs form a ternary complex with the target protein and E3 and consequently promote target ubiquitination and degradation[193].

Salami *et al.*[194] published a study in 2018 that aimed to assess whether ARCC-4, a low-nanomolar PROTAC ARD, was better at targeting AR signaling in CRPC cells than the currently approved competitive antagonist ENZ. Interestingly, ARCC-4 promoted the degradation of about 95% of cellular ARs, especially the clinically relevant AR with point mutations resistant to ENZ. Moreover, ARCC-4 induced apoptosis and maintained its antiproliferative effect in a hyperandrogenic environment that impedes ENZ activity. This study showed promising results where PROTAC-mediated AR degradation might overcome the mCRPC AR-dependent

mechanism of drug resistance[194].

To date, several PROTACs reached clinical trials in different types of cancer, including ARV-110 in mCRPC. The oral and bioavailable ARV-110 was tested earlier *in vitro* and *in vivo*, where it led to the degradation of more than 95% of ARs in the tested PCa cell lines and demonstrated antitumor effects in both ENZ-naïve and -resistant PCa xenograft models. ARV-110, similar to ARCC-4, promoted the degradation of clinically relevant AR mutants and maintained its activity even in a milieu with high androgen levels. The first-in-human, Phase I clinical trial (NCT03888612) testing ARV-110 in mCRPC patients who received more than two prior treatments was performed. This trial is still ongoing; however, early data show that ARV-110 has a reasonable safety profile and a promising antitumor activity in mCRPC[153].

The results from these preclinical and clinical trials, among others, show that ARD might be a novel alternative therapeutic option to treat mCRPC.

PI3K pathway inhibitors

As mentioned above, loss of PTEN and the atypical activation of the PI3K signaling network are one of the possible mechanisms driving mCRPC growth. This fact supported the development of several PI3K pathway inhibitors including ipatasertib (Ipat) (GDC-0068), which is an oral, bioavailable, AKT non-ATP-competitive inhibitor that impedes the PI3K/AKT pathway and subsequently tumorigenesis[195].

Two clinical trials, a Phase Ib/II (NCT01485861) involving patients who formerly received DOC and an ongoing Phase III trial (NCT03072238) with treatment-naïve patients, tested the combination of Ipat and ABI in mCRPC. In general, both studies demonstrated enhanced antitumor effects when combining Ipat with ABI compared to ABI monotherapy, with increased benefit for patients with PTEN loss[154-156]. Thus, the results of those trials suggest that a combination of AKT and AR signaling inhibitors might provide a promising therapeutic approach to treating men with mCRPC with a poor prognosis due to PTEN loss.

CONCLUSION

mCRPC remains a challenge for PCa disease management. AR signaling comprises a fundamental role in PCa pathogenesis even in the advanced androgen-insensitive stages. AR signaling, along with other various molecular pathways, is subjected to diverse modifications and aberrations that can be used as biomarkers to personalize treatment for mCRPC patients and overcome drug resistance to the standard therapy. Thus, further understanding of the mechanisms mediating drug resistance in mCRPC is crucial for identifying future targeted therapeutic modalities.

DECLARATIONS

Authors' contributions

Conceptualized, outlined, and drafted the review: Abou-Kheir W, Mukherji D

Made substantial contributions to study design, drafting the paper, generating tables and figures: Yehya A, Ghamlouche F, Zahwe A

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Cancer Drug Resistance

Review

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Persistent EGFR/K-RAS/SIAH pathway activation drives chemo-resistance and early tumor relapse in triple-negative breast cancer

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Abstract

Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype. It disproportionately affects BRCA mutation carriers and young women, especially African American (AA) women. Chemoresistant TNBC is a heterogeneous and molecularly unstable disease that challenges our ability to apply personalized therapies. With the approval of immune checkpoint blockade (ICB) for TNBC, the addition of pembrolizumab to systemic chemotherapy has become standard of care (SOC) in neoadjuvant systemic therapy (NST) for high-risk early-stage TNBC. Pembrolizumab plus chemotherapy significantly increased the pathologic complete response (pCR) and improved event-free survival in TNBC. However, clinical uncertainties remain because similarly treated TNBC partial responders with comparable tumor responses to neoadjuvant therapy often experience disparate clinical outcomes. Current methods fall short in accurately predicting which high-risk patients will develop chemo-resistance and tumor relapse. Therefore, novel treatment strategies and innovative new research initiatives are needed. We propose that the EGFR-K-RAS-SIAH pathway activation is a major tumor driver in chemoresistant TNBC. Persistent high expression of SIAH in residual tumors following NACT/NST reflects that the EGFR/K-RAS

pathway remains activated (ON), indicating an ineffective response to treatment. These chemoresistant tumor clones persist in expressing SIAH ($SIAH^{High/ON}$) and are linked to early tumor relapse and poorer prognosis. Conversely, the loss of SIAH expression ($SIAH^{Low/OFF}$) in residual tumors post-NACT/NST reflects EGFR/K-RAS pathway inactivation (OFF), indicating effective therapy and chemo-sensitive tumor cells. $SIAH^{Low/OFF}$ signal is linked to tumor remission and better prognosis post-NACT/NST. Therefore, SIAH is well-positioned to become a novel tumor-specific, therapy-responsive, and prognostic biomarker. Potentially, this new biomarker ($SIAH^{High/ON}$) could be used to quantify therapy response, predict chemo-resistance, and identify those patients at the highest risk for tumor relapse and poor survival in TNBC.

Keywords: Triple-negative breast cancer (TNBC), chemo-resistance, seven in absentia (SINA) and human homologs of SINA (SIAH) E3 ligase, ubiquitin-mediated proteolysis, EGFR/K-RAS/SIAH pathway activation in TNBC, neoadjuvant chemotherapy prognosis, patient risk stratification, detection of chemo-resistance, precision quantification of therapy efficacy, and treatment optimization

INTRODUCTION

Triple-negative breast cancer

Triple-negative breast cancer (TNBC) represents 15%-20% of all breast cancers diagnosed in the United States, and it is characterized by the absence of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2)[1-5]. https://seer.cancer.gov/statfacts/html/breast.html. TNBC is the most aggressive subtype of breast cancer, and it disproportionately affects *BRCA1* mutation carriers and young women, especially those with Western African ancestry[6-13]. This molecular subtype is nearly twice as common in African American women (AA) than in Caucasian women[6,8,9,14-17]. TNBC is a genetically diverse, highly heterogeneous, and molecularly unstable disease, which challenges our ability to tailor effective individualized treatments for patients[10,18]. TNBC has unique and aggressive tumor biology, and it constitutes a major health threat with the worst prognosis and the highest mortality of all breast cancer subtypes[10,19,20]. Looking more closely, one in three patients with high-risk early-stage TNBC will develop tumor relapse, which typically occurs within the first three years of initial diagnosis; only a third of women with locoregional TNBC will survive their disease; and of those with metastatic TNBC, less than 1 in 9 will survive their disease[5,10,21-23]. TNBC has a 5-year overall survival (OS) of 78.5%, and the 5-year survival rates for localized, regional, and metastatic diseases are 91.2%, 65.4%, and 12.2%, respectively, which is the worst among the major molecular subtypes in breast cancer (https://seer.cancer.gov/explorer/application.html?site=623&data_type=4&graph_type=5&compareBy=stage&chk_stage_101=101&chk_stage_104=104&chk_stage_105=105&chk_stage_106=106&series=9&hdn_sex=3&race=1&age_range=1&advopt_precision=1&advopt_show_ci=on&advopt_display=2). Thus, the dismal prognosis, chemo-resistance, and high mortality of regional and metastatic TNBC highlights a critical unmet need for the development of improved therapies and the discovery of reliable prognostic biomarkers such as SIAH, which can single out the highest risk patients at the first-line neoadjuvant setting, identify chemoresistant tumors that are difficult to treat and prone to develop early relapse, optimize effective treatment sequences, and select the best combinational strategies for better clinical outcomes and prolonged survival.

Standard treatment regimens in TNBC

Standard chemotherapy remains the backbone of systemic therapy in TNBC[20,24-28]. https://www.cancer.org/cancer/breast-cancer/treatment/treatment-of-triple-negative.html. Neoadjuvant chemotherapy (NACT) was the previous standard of care (SOC) to treat high-risk and locally advanced TNBC prior to July 26, 2021[10,29]. The addition of immune checkpoint blockade (ICB) to chemotherapy is now the current SOC for neoadjuvant systemic therapy (NST) to treat high-risk early-stage TNBC. Immuno-oncology (IO) therapy is an exciting scientific breakthrough in the treatment of TNBC[30-32].

Immunotherapy that targets programmed death receptor-1 (PD-1) has shown great promise in treating a subset of TNBC patients in combination with chemotherapy[33,34]. Pembrolizumab plus chemotherapy significantly improved the pCR rates in high-risk early-stage TNBC in the neoadjuvant setting[35,36]. As shown in the KEYNOTE-522 trial, neoadjuvant pembrolizumab plus chemotherapy led to an improved pCR rate (65%) in high-risk early-stage TNBC[35]. Notably, the TNBC pIR patients with residual disease at the time of surgery seemed to benefit the most from the addition of IO-therapy in the neoadjuvant and adjuvant settings[37]. Surprisingly, the 3-year event-free survival (EFS) benefit associated with pembrolizumab was independent of PD-L1 expression in high-risk early-stage TNBC[35]. Based on the I-SPY 2 trial, the pCR rate doubled to 60% when pembrolizumab was added to standard chemotherapy to treat stage II/III TNBC patients with T2/N1 or higher stage tumors[30,36]. In contrast, as reported in KEYNOTE-355, KEYNOTE-119, Impassion130, and Impassion131, PD-(L)1-targeted immunotherapies plus chemotherapy have shown only modest survival benefit in PD-L1-positive TNBC in advanced and metastatic settings[33,34,38]. At the same time, unfortunately, the grade 3 or 4 treatment-related adverse events were significantly increased in response to the new IO-regimens[39].

Unmet needs in TNBC

As more and more TNBC patients are treated with pembrolizumab plus chemotherapy in both neoadjuvant and/or metastatic settings, ICB resistance is evidently emerging, and serious adverse side effects were reported in a subset of TNBC patients[35,38]. Despite the benefit of this newly FDA-approved IO-therapy, 30%-44% of high-risk early-stage TNBC patients and 60%-70% of PD-L1-positive metastatic TNBC patients who receive the IO-therapy did not show any objective improvement[35,38]. Without a proper guide, pembrolizumab plus chemotherapy is often administered "blindly" in the neoadjuvant setting following the newly FDA-approved standard IO-regimens to treat high-risk early-stage TNBC. How to maximize the current SOC chemo- and IO-therapy in combination while limiting chemo- and IO-resistance, and minimizing the side effects of immunotherapy is a difficult problem and an unmet need for a large number of TNBC patients.

Pathology following completion of NACT/NST, with or without immunotherapy (pembrolizumab), produces a binary response: pathologic complete response (pCR) or pathologic incomplete response (pIR)[40,41]. pCR is a reliable prognostic marker that correlates with tumor remission and long-term survival, whereas pIR is associated with an increased risk of early tumor relapse and poor prognosis[36,42-47]. Incomplete responders can be further classified by the residual cancer burden (RCB classes I-III); the higher the RCB classification, the higher the likelihood of tumor relapse and mortality[21,40,41,48-51]. TNBC patients with high-risk and high-grade residual disease are now commonly treated with additional adjuvant chemotherapy, including capecitabine, which may be combined with immunotherapy (pembrolizumab) post-operatively[35,36,38,52-54]. Clinical uncertainties remain, because although many TNBC patients with the identical clinical and pathological tumor stages by the American Joint Committee on Cancer (AJCC) TNM classification, and similar residual cancer burden (RCB) after a non-pCR (pIR) diagnosis post-NACT/NST, will often experience disparate clinical outcomes and survival[55,56]. Current methods to stratify these high-risk patients fall short in predicting the risk of tumor recurrence and forecasting survival. There is no reliable prognostic biomarker that can be used to predict with certainty and molecular precision which RCB (I-II-III) tumors will stay in remission and which ones will relapse rapidly[3]. Few therapeutic agents, alone or in combination, are effective at eradicating chemoresistant and metastatic TNBC[57-60]. Therefore, the development of a new tumor-specific biomarker that can be used to stratify high-risk TNBC patients in the first-line neoadjuvant setting, quantifying treatment efficacy in real time in the clinical setting is essential. Additionally, utilizing this same biomarker to detect the emergence of chemoresistant tumor clones at a single tumor cell resolution, forecast risk for early tumor relapse, and predict patient survival would equip us with a new therapy-responsive prognostic biomarker to quantify, guide, and treat TNBC more

effectively[3].

Chemo-resistance in TNBC

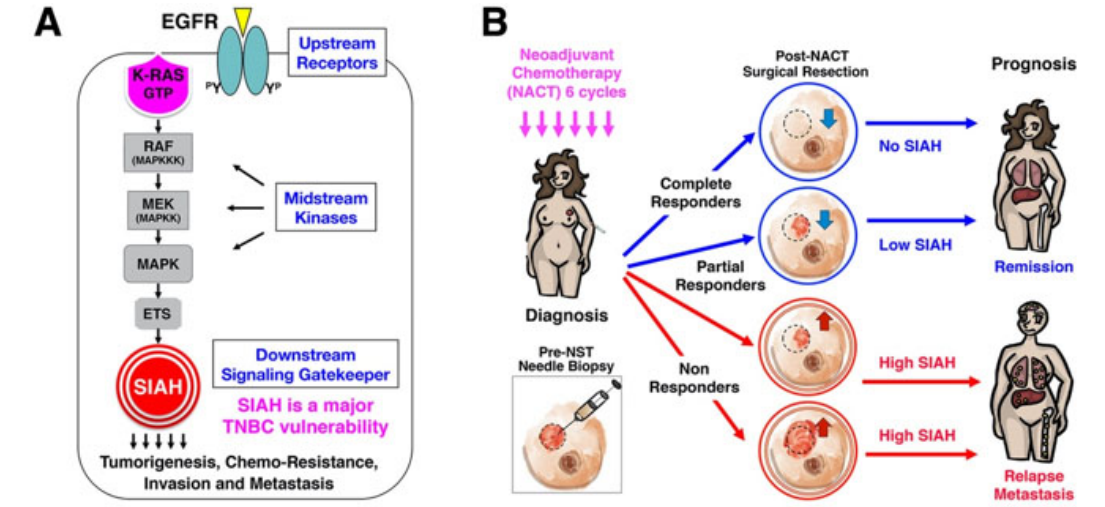
Chemo-resistance is a vexing problem and a major life-threatening feature of TNBC[25,57,61]. Activation of multiple signaling pathways, context-dependent compensatory pathway cross-talk, synergy, antagonism, and signaling network "rewiring" are all implicated in the development of chemoresistant phenotypes in TNBC. These include Wnt/β-catenin, Notch, Hedgehog, NFκB, PI3K/mTOR, Hippo/YAP, JAK/Stat, TGFβ, hypoxia, p53 loss of function and BRCA mutations, altered metabolism, and increased transporter and efflux pump activity[25,57,62-66]. Single-cell sequencing has revealed that plasticity, heterogeneity, rapid molecular evolution of innate and acquired chemo-resistance, cellular senescence, and dynamic remodeling of epithelial-mesenchymal transition (TME)/mesenchymal-epithelial transition (MET) states of tumor-initiating cells or cancer stem cells in TNBC contribute to cancer recurrences[49,62,67-71]. These aforementioned topics have been reviewed extensively in the TNBC literature. Here, our discussion will focus on persistent activation of the EGFR/K-RAS/MAPK/SIAH pathway, which drives chemo-resistance, early tumor relapse, and high mortality in TNBC[3,72].

Persistent EGFR/K-RAS/MAPK/SIAH pathway activation drives TNBC malignancy

Genomic landscape studies indicate that EGFR/K-RAS/MAPK pathway activation is a major impetus driving TNBC malignancy, early tumor relapse, local invasion, and metastatic spread[73,74]. Aberrant EGFR/K-RAS/MAPK/SIAH pathway activation is highly prevalent in chemoresistant, recurrent, locally advanced, and metastatic TNBC[3,75-78]. Heightened EGFR/K-RAS/MAPK activation has multiple deleterious effects on the tumor/tumor microenvironment (TME), which is associated with decreased tumor-infiltrating lymphocytes (TIL) detection in TNBC and is correlated with increased metastases and poor prognosis in breast cancer[73,74,78]. With the new FDA-approved chemo- and immunotherapy combination to treat high-risk early-stage TNBC, it is important to maximize the therapeutic benefit and identify chemoresistant tumor cells as early as possible, but also minimize the adverse toxicities and immune side-effects of these IO-combination therapies in the neoadjuvant and adjuvant settings.

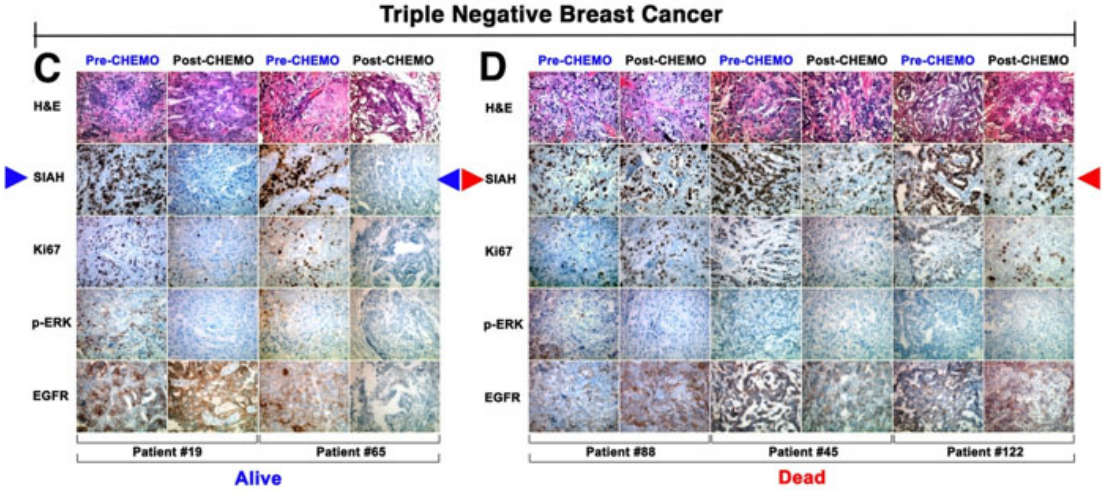
SIAH is the most conserved downstream signaling gatekeeper in the EGFR/K-RAS/MAPK signaling pathway

Due to the extraordinary conservation of EGFR/RAS/MAPK/SIAH signaling pathway across metazoan species, the molecular insights and core principles gleaned from *Drosophila* EGFR/RAS/SINA studies have shed light on the evolutionarily conserved principles and fundamental aspects of mammalian EGFR/K-RAS/MAPK/SIAH signaling pathway, and guided anti-EGFR/K-RAS/MAPK/SIAH drug development in human cancer[77,79-85]. As a RING-domain E3 ubiquitin ligase, the human homologs of SINA (SIAH) or *Drosophila* Seven-In-Absentia (SINA) are the most downstream gatekeeper and the most evolutionarily conserved signaling component in the EGFR/K-RAS/MEK/MAPK pathway identified thus far [Figure 1A, 1E and 1F][72,77,82-88]. Due to its conserved signaling gatekeeper function as a major network bottleneck, $SIAH^{ON/OFF}$ expression is well-positioned to serve as a direct readout of tumor-driving EGFR/K-RAS/MEK/MAPK pathway activation (ON)/inactivation (OFF) in TNBC [Figure 1B][76,77,85,89]. $SIAH^{Low/OFF}$ in TNBC post-NACT correlates with tumor remission, effective therapy, and good prognosis [Figure 1C], whereas $SIAH^{High/ON}$ in TNBC post-NACT correlates with early relapse, ineffective therapy, and poor survival [Figure 1D][76]. Therefore, SIAH is likely to be an excellent prognostic biomarker to stratify incomplete responders in the first-line neoadjuvant setting[3,76,89]. SIAH may be used to identify chemoresistant tumor cells as early as possible in the neoadjuvant setting as a therapy-responsive biomarker in order to identify the difficult-to-treat cancers at the highest-risk for early relapse and treatment-resistance. Furthermore, we propose that SIAH can be used to augment residual cancer burden (RCB I-III) classification in quantifying the efficacy of SOC treatment regimens, detect chemoresistant tumor clones,



EGFR/K-RAS/SIAH pathway activation drives early tumor relapse and TNBC malignancy

Standard of care (SOC) neoadjuvant chemotherapy (NACT) to treat high-risk and locally advanced TNBC



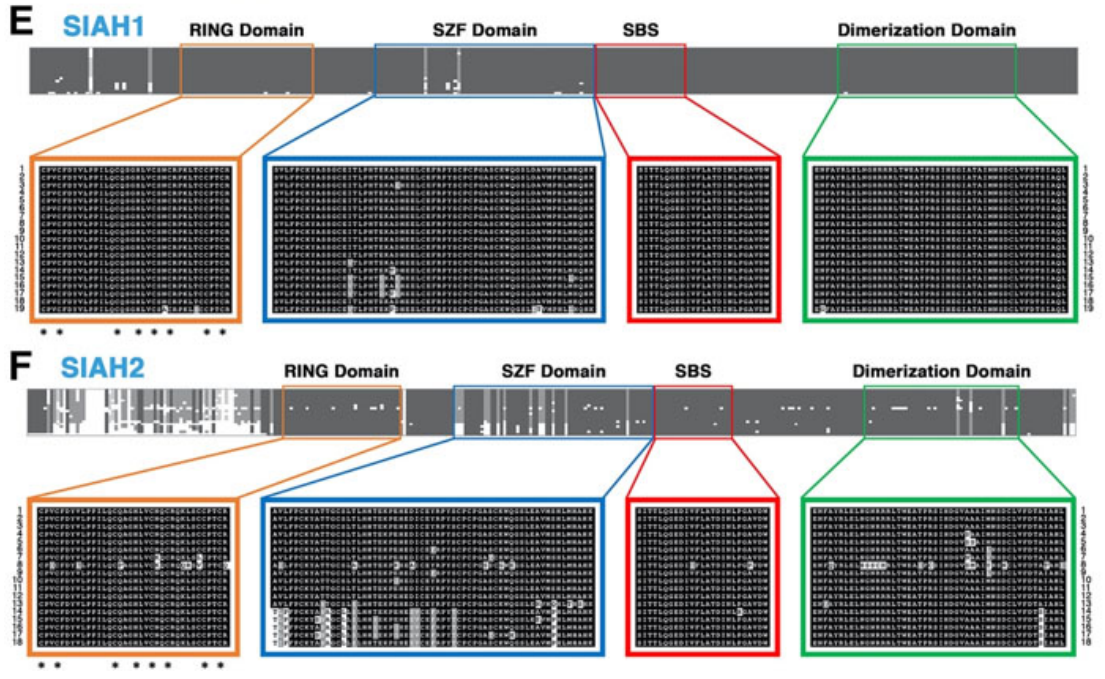


Figure 1. SIAH is the most conserved downstream signaling gatekeeper in the EGFR/K-RAS/SIAH pathway, whose persistent activation is driving TNBC malignancy, tumor relapse, and metastasis. (A) SIAH is the most evolutionarily conserved and the most downstream signaling module identified in the EGFR/K-RAS signaling pathway thus far. (B) Loss of SIAH expression ($SIAH^{Low/OFF}$) after effective NACT is correlated with EGFR/K-RAS pathway inactivation and tumor regression/remission, whereas persistent SIAH expression ($SIAH^{High/ON}$) after ineffective NACT is correlated with EGFR/K-RAS pathway activation and tumor progression/early relapse. Persistent high SIAH expression ($SIAH^{High/ON}$) in high-risk residual tumors post NACT is correlated with EGFR/K-RAS pathway activation, chemo-resistance, and early tumor relapse. (C-D) TNBC tumors were stained with H&E, SIAH, Ki67, phospho-ERK, and EGFR. SIAH outperforms Ki67. SIAH is prognostic and Ki67 is not prognostic in NACT-treated high-risk and locally advanced breast cancer. We found that $SIAH^{Low/OFF}$ post-NACT correlates with tumor remission and prolonged survival (Alive at 5 years) (C). We found that persistent $SIAH^{High/ON}$ expression in residual tumors post-NACT is associated with tumor relapse and poor survival (Dead before3-5 years) (D). (E) SIAH1 and (F) SIAH2 are extraordinarily conserved across metazoan species. Conclusion: We found that $SIAH^{ON/OFF}$ expression is a binary code that reflects tumor-driving EGFR/K-RAS/SIAH pathway activation^{ON}/inactivation^{OFF} in TNBC primary and residual tumors As such, SIAH is strategically well positioned to become a new TNBC target, and a new tumor-specific, therapy-responsive, and prognostic biomarker to risk-stratify pIR patients, detect the emergence of treatment-refractory tumors, quantify NACT/NST efficacy, augment RCB classifications, forecast early relapse, and predict patient survival in real time in the clinic. SIAH: Human homologs of *Drosophila* Seven In Absentia (SINA); NACT: neoadjuvant chemotherapy; RCB: residual cancer burden; TNBC: triple-negative breast cancer.

forecast early tumor relapse, and predict patient survival. Having this accuracy in real time allows the clinician to pivot with precision to select more effective drugs and optimize the sequence of combination therapies for treatment-refractory TNBC [Figure 1][3,76,77,85].

The $SIAH^{ON/OFF}$ binary code reflects this major tumor-driving EGFR/K-RAS/MAPK pathway activation (ON) and inactivation (OFF) in TNBC

Supported by strong evidence in developmental, evolutionary, and cancer biology, we hypothesize that persistent EGFR–K-RAS–SIAH pathway activation is a major tumor-driving force in TNBC, and that SIAH is a new tumor-specific, therapy-responsive, and prognostic biomarker for patient risk stratification, therapy quantification, and treatment optimization[76,77,82-85,89]. We propose that the persistent high expression of SIAH ($SIAH^{High/ON}$) post-NACT/NST reflects tumor-driving EGFR/K-RAS/MAPK pathway activation (ON), resulting in tumor progression, immuno-suppression, and chemo-resistance, versus the loss of SIAH expression ($SIAH^{Low/OFF}$) post-NACT/NST reflects this tumor-driving pathway is inactivated (OFF), resulting in tumor regression, immune responsiveness, and chemo-sensitivity[3,76]. This new tumor-specific, therapy-responsive, and prognostic $SIAH^{ON/OFF}$ binary code can potentially be used to identify those TNBC pIR patients at the highest risk for early tumor relapse, detect multidrug-resistant residual tumor clones in real time, combine and guide precise therapies in the first-line setting.

Clinical utility of SIAH as a tumor-specific, therapy-responsive, and prognostic biomarker for risk stratification, early relapse, and survival prediction in TNBC

We generated an anti-SIAH monoclonal antibody for tumor IHC/IF/FACS staining[82,83]. Our pilot study of 57 NACT-treated TNBC patients with residual disease showed that $SIAH^{Low/OFF}$ correlates with tumor remission and good prognosis [Figure 1C]. For those with $SIAH^{High/ON}$ in their residual tumors, it predicts chemoresistant tumor cells/clones, early tumor relapse, and poor prognosis post-NACT/NST [Figure 1D][76]. The prognostic impact of SIAH expression seemed to be far superior to that of Ki67 and phospho-ERK in NACT/NST-treated breast cancer[76]. SIAH could be used to risk stratify incomplete responders, identify chemo- and IO-resistance, quantify therapy efficacy, and predict relapse and survival [Figure 2A]. $SIAH^{Low/OFF}$ in TNBC residual tumors reflects that the EGFR/K-RAS/MAPK/SIAH pathway is OFF, and indicates chemo-sensitivity, effective NACT/NST, and good prognosis after surgery [Figure 2B]. $SIAH^{High/ON}$ in TNBC residual tumors reflects that the EGFR/K-RAS/MAPK/SIAH pathway is ON, which indicates chemo-resistance, ineffective NACT/NST, and/or the need for additional adjuvant therapies to prevent progressive disease and early tumor relapse [Figure 2C]. Our studies have demonstrated that SIAH offers a tumor-specific, therapy-responsive, and prognostic biomarker in TNBC with high molecular precision and full dynamic range (0%-100%)[76,89]. Therefore, $SIAH^{ON/OFF}$ expression can be used to identify chemoresistant

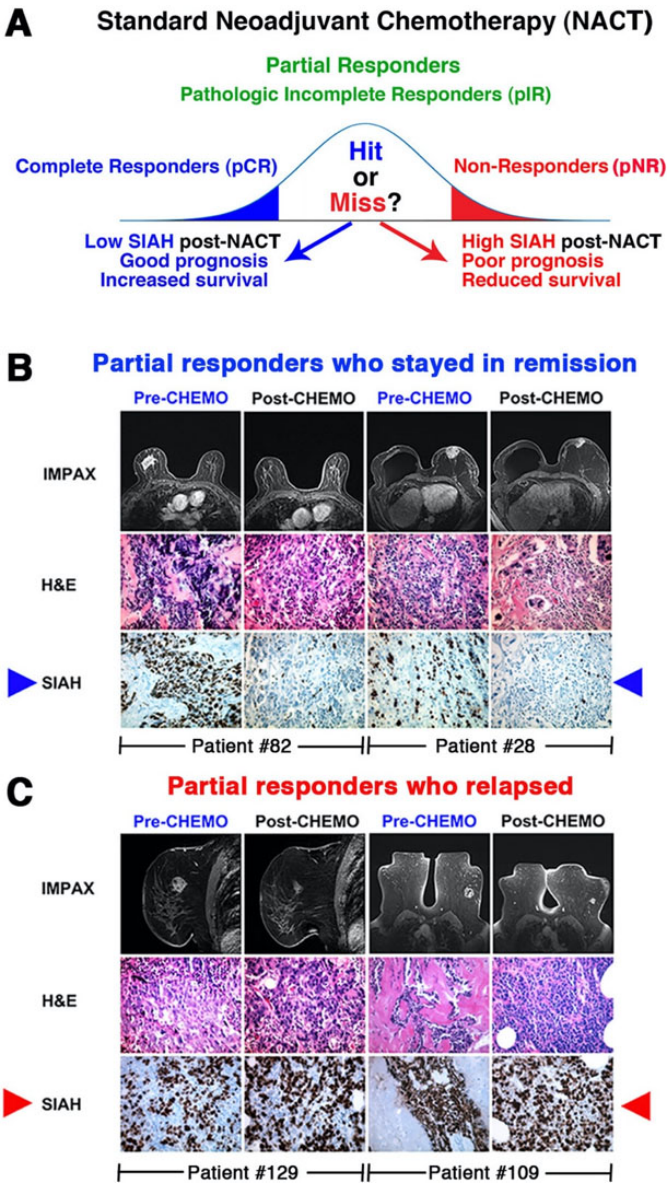


Figure 2. SIAH marks proliferating tumor cells at a single-cell resolution, and SIAH is a therapy-responsive and prognostic biomarker that can be used to risk-stratify incomplete responders in NACT/NST-treated high-risk and locally advanced breast cancer. IMPAX stands for the IMPAX digital mammography, a powerful diagnostic platform for breast imaging at the Sentara Breast Centers. (A) pCR is a good prognostic marker associated with long-term survival post-NACT/NST. However, for the pIR patients with residual disease, additional tools are needed to distinguish which patients are at high risk for early tumor relapse and thus who may need additional adjuvant chemotherapies. (B) The pIR patients with no or low SIAH expression in residual tumors post-NACT/NST stayed in remission and have prolonged survival (examples: patients #82 and #28). (C) In contrast, the pIR patients with persistent high SIAH expression in residual disease post-NACT/NST developed early relapse and succumbed to their chemoresistant and metastatic diseases (examples: patients #109 and #129). Conclusion: We propose that persistent high SIAH expression in residual tumors is associated with early tumor relapse and poor prognosis, while no or low SIAH expression in residual tumors is associated with tumor remission and good prognosis post-NACT/NST. SIAH: Human homologs of *Drosophila* Seven In Absentia (SINA); Pir: pathologic incomplete response; NACT: neoadjuvant chemotherapy; NST: neoadjuvant systemic therapy; PCR: athologic complete response.

tumor clones, differentiate partial responders, forecast tumor relapse, and predict patient survival in NACT-treated breast cancer[76,89]. Moreover, we have shown that $SIAH^{ON/OFF}$ expression is a direct readout of EGFR/K-RAS/MAPK pathway activation (ON)/inactivation (OFF)[3,76,77,84,85]. Hence, studying the "ON/OFF" of the major tumor-driving EGFR/K-RAS/MAPK/SIAH pathway may represent an opportunity to risk-

stratify TNBC patients before and after NACT/NST (IO-therapy). As a binary biomarker with high tumor specificity and detection sensitivity as well as a full dynamic range (0%-100%), we propose to validate that SIAH is a powerful new prognostic biomarker that can be used to risk-stratify patients, detection of chemo-resistance, quantify NACT/NST efficacy, forecast early tumor relapse, and predict patient survival in TNBC[72,76,77,82,83].

CONCLUSION

Focusing on EGFR/K-RAS/MAPK/SIAH pathway activation as a major tumor driver in TNBC to risk-stratify patients and detect chemo-resistance may represent a significant step forward. In our pilot study, we found that all TNBC primary tumors are highly proliferative, heavily decorated with SIAH, and the median SIAH expression in untreated TNBC tumors was 70% in both lymph node (LN) positive and LN-negative subsets[76]. In contrast, 30% of patients with residual disease displayed persistent high SIAH expression and had high relapse rates and poor outcomes[76]. As a tumor-specific, therapy-responsive, and prognostic biomarker in TNBC, SIAH has a full dynamic range, high sensitivity, high specificity, and molecular precision and could be used to risk-stratify patients and detect chemoresistant tumor cells in the first-line neoadjuvant settings. Ultimately, we hope to translate our findings (SIAH as a new prognostic biomarker) for clinical use to facilitate early detection of ineffective therapy in the first-line neoadjuvant setting, detect chemoresistant tumor cells at a single tumor cell resolution, augment RCB I-II-III classifications with high-precision, accurately calculate the risk of early tumor relapse and predict long-term survival in TNBC. If successful, we can use this new, dynamic, therapy-responsive, interactive, and tumor-specific biomarker, SIAH, to address the unmet need of identifying chemoresistant TNBC, and risk-stratify pIR patients by tumor relapse and poor prognosis in the first-line neoadjuvant setting. By leveraging the tumor-driving EGFR/K-RAS/SIAH pathway activation (ON)/inactivation (OFF) in TNBC, we hope to differentiate TNBC pIR patients by correlating $SIAH^{High}$ (high-risk) versus $SIAH^{Low}$ (low-risk) expression in residual tumors post-NACT/NST. This precision biomarker may also be used to detect treatment disparity amongst incomplete responders, forecast early tumor relapse, and predict survival. The next steps should include the successful executions of several independent large-scale multicenter biomarker validation studies leading to FDA approval of SIAH as a new tumor-specific, therapy-responsive, and prognostic biomarker for TNBC risk stratification, detection of chemo-resistance, therapy quantification in real time, treatment optimization in the clinic. Importantly, SIAH is a strategically positioned cancer target for us to develop a new anti-TNBC targeted therapy to eradicate multidrug-resistant, undruggable, and incurable TNBC malignancy in the future.

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Authors' contributions

Formulated the original idea, novel concept, central hypothesis, data collection, experimental execution, designed the figures and wrote the early drafts: Tang AH
Provided the seed money and visionary leadership to support this study since its conception: Hoefer RA
Advised, guided, contributed, and supported this original idea, novel concept, and large-scale biomarker validation studies: Hoefer RA, Guye ML, Bear HD
All authors have met the four criteria of the authorship requirements as listed by the ICMJE.
All authors have made important contribution, improved the intellectual content, and added their scientific and clinical expertise to strengthen, augment, and support this work.
All authors have read, edited, and approved the finalized manuscript for publication.
All authors have contributed to writing, editing, revising, proofreading, and rewriting of this manuscript.

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Active IRB approval: The Institutional Review Boards (IRB) approval is in place to conduct this study.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Base excision repair accessory factors in senescence avoidance and resistance to treatments

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Abstract

Cancer cells, in which the RAS and PI3K pathways are activated, produce high levels of reactive oxygen species (ROS), which cause oxidative DNA damage and ultimately cellular senescence. This process has been documented in tissue culture, mouse models, and human pre-cancerous lesions. In this context, cellular senescence functions as a tumour suppressor mechanism. Some rare cancer cells, however, manage to adapt to avoid senescence and continue to proliferate. One well-documented mode of adaptation involves increased production of antioxidants often associated with inactivation of the *KEAP1* tumour suppressor gene and the resulting upregulation of the NRF2 transcription factor. In this review, we detail an alternative mode of adaptation to oxidative DNA damage induced by ROS: the increased activity of the base excision repair (BER) pathway, achieved through the enhanced expression of BER enzymes and DNA repair accessory factors. These proteins, exemplified here by the CUT domain proteins CUX1, CUX2, and SATB1, stimulate the activity of BER enzymes. The ensued accelerated repair of oxidative DNA damage enables cancer cells to avoid senescence despite high ROS levels. As a by-product of this adaptation, these cancer cells exhibit increased resistance to genotoxic treatments including ionizing radiation, temozolomide, and cisplatin. Moreover, considering the intrinsic error rate associated with DNA repair and translesion synthesis, the elevated number of oxidative DNA lesions caused by high ROS leads to the accumulation of mutations in the cancer cell population, thereby contributing to tumour heterogeneity and eventually to the acquisition of resistance, a major obstacle to clinical treatment.

Keywords: Base excision repair, reactive oxygen species, DNA repair accessory factor, oxidative DNA damage, resistance to treatment, tumour heterogeneity, acquisition of resistance

BASE EXCISION REPAIR

Overview

Base excision repair (BER) repairs most base lesions including alkylated, deaminated and oxidized bases, as well as apurinic/apyrimidinic (AP) sites [Figure 1][1]. This pathway is initiated by one of many DNA glycosylases, which recognizes a specific base lesion and cleaves the N-glycosylic bond linking the altered base to the DNA backbone to produce an apyrimidinic/apurinic site (AP site)[1,2]. In mammals, AP sites are targeted by the AP endonuclease 1, APE1, which incises the DNA backbone 5' to the AP site to generate a single-strand break with a 5'-deoxyribose phosphate (dRP)[3]. In addition, DNA glycosylases for oxidized bases are also endowed with an AP/lyase activity that generates a single-strand nick 3' to the AP site. OGG1 and NTHL1 generate the single-strand nick through beta elimination, while NEIL1 and NEIL2 do this by beta delta elimination (reviewed in[4]). 5' or 3' end processing of the resulting single-strand breaks is then performed by DNA Pol β, APE1, or PNKP, and repair synthesis and ligation are accomplished by the short-patch or long-patch pathways[5-7]. In short-patch repair, DNA Pol β (Pol β) adds a single base and removes the 5'- dRP to allow ligation[8]. In long patch repair, 2 to 13 bases are synthesized by Pol β or δ/ε, thereby generating a displaced strand that is cleaved by the flap structure-specific endonuclease FEN1 prior to ligation[1,9].

The importance of BER is illustrated by its complex implications in cancer. On the one hand, inherited as well as somatic mutations in some BER genes were found in various cancers and in some cases have been linked to particular mutation signatures. On the other hand, overexpression of BER enzymes has been reported in many tumours and cancer cell lines and has been associated with increased resistance to genotoxic treatments.

Germ line and somatic mutations in BER genes that predispose to cancer

The role of Pol β in maintaining genome integrity is illustrated by the fact that mouse embryo fibroblasts from Pol β-null mice exhibit a 1.6-fold increase in spontaneous mutation frequency[10]. However, it is important to consider that the role of Pol β in the repair of endogenous DNA lesions does contribute to the acquisition of mutations. The average Pol β error rate was estimated at 7×10^{-4} for 12 possible base substitution errors and at 3 to 9×10^{-4} for single base deletions[11]. In a diploid mammalian cell, there are approximately 30,000 endogenous DNA base lesions per day[12] (and table 2.1 in[13]). The term "endogenous" here means that these lesions are caused by normal metabolism in contrast to exogenous sources of DNA damage. Considering a Pol β error rate of at least 1/1000, we can estimate that 30 mutations per cell are acquired every day as a result of BER activity. This said, inherited and somatic mutations that alter the function of BER enzymes increase the risk of cancer, and for this reason, BER is considered to function as a tumour suppressor mechanism.

Approximately 30% of tumours analyzed exhibit a mutation in the *Pol β* gene, and there is evidence that these Pol β variants can contribute to tumour development[14]. The Pol β^{K289M} variant was found to cause a 2.5-fold increase in mutation frequency[15]. Moreover, stable expression of this Pol β^{K289M} variant, as well as another variant, Pol β^{I260M}, was found to induce a transformed phenotype in mouse cells, as judged from focus formation on a monolayer and a soft-agar assay[16].

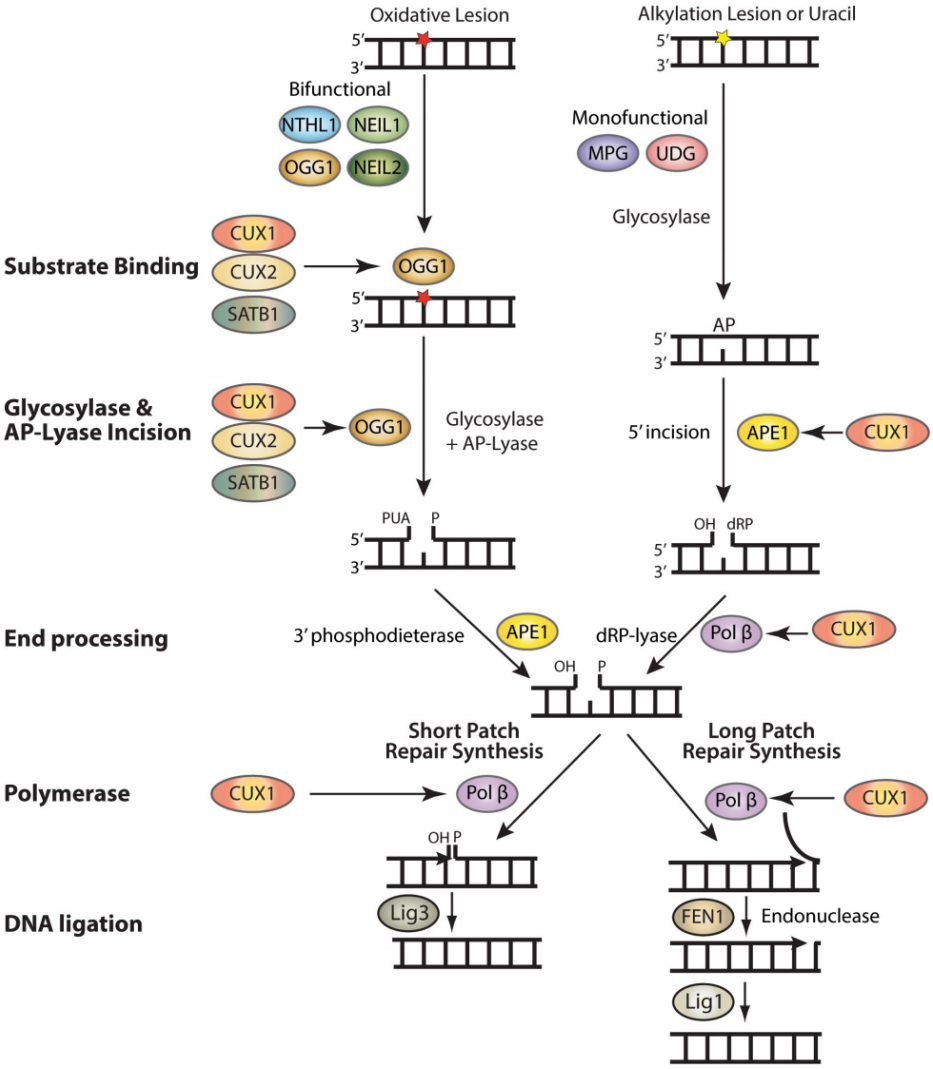

Figure 1. Auxiliary factors stimulate the enzymatic activities of several BER enzymes. BER is initiated by the removal of a damaged base by a glycosylase which creates an apyrimidinic or apurinic site (AP site). Bifunctional glycosylases such as NTHL1, OGG1, NEIL1, and NEIL2 have an AP-lyase activity and can introduce a single-strand break. APE1 then removes the phosphor-α,β-unsaturated aldehyde (PUA) to create a single-strand break with a 3′-OH and a 5′-phosphate. In the case of MPG and UDG, both monofunctional glycosylases, APE1 will cleave the backbone, leaving a single-strand break with a 3′dRP that will be removed by Pol β. In short patch repair, Pol β inserts the missing nucleotide and Lig3 seals the break. In long patch repair, Pol β inserts multiple nucleotides, FEN1 cleaves the displaced strand, and Lig1 seals the break. The CUT domain proteins CUX1, CUX2, and SATB1 stimulate the binding of OGG1 to 8-oxo-deoxyguanine as well as its glycosylase and AP-lyase enzymatic activities. The CUT domains of CUX1 have also been shown to stimulate the 5′-incision activity of APE1 as well as the dRP-lyase, DNA polymerase, and strand-displacement activities of DNA Pol β.

In addition to somatic mutations, some germline mutations in BER proteins predispose to cancer. MUTYH-associated polyposis (MAP) was first described when two inherited germline mutations in the *MUTYH* gene (Y165C and G382D) were identified in three siblings with clinical symptoms of familial adenomatous polyposis (FAP), an inherited condition that predisposes to colorectal cancer (CRC)[17]. *MUTYH* is the human homologue of MutY, a DNA glycosylase that removes adenine from 8-oxoG:A base pairs, preventing G:C to T:A transversions (reviewed in[18]). The adenine glycosylases encoded by the $MUTYH^{Y165C}$ and $MUTYH^{G382D}$ mutants displayed much reduced activity[17]. Further investigations confirmed that inherited *MUTYH* mutations increase the risk of colorectal polyposis as well as other cancers including duodenal carcinoma, bladder, ovarian, and skin cancer[19-23].

More recently, a homozygous nonsense mutation in the *NTHL1* DNA glycosylase gene was also identified in individuals with multiple colonic adenomas from three unrelated families, suggesting a new BER-associated predisposing condition for colorectal cancer[24]. This mutation confers a highly penetrant predisposition to adenomatous polyposis and is also associated with other benign and malignant lesions[25]. The NTHL1-associated polyposis (NAP) and findings of NTHL1 variants and altered regulation in tumors, provide another evidence on the role of BER in maintaining genomic stability and preventing cell transformation[26]. Note that the patterns of somatic mutations associated with MAP and NAP are distinct and are related to specific mutation signatures[25,27,28].

COOPERATING EVENTS IN TUMOUR DEVELOPMENT

The notion of multi-step carcinogenesis arose from early experiments with chemical carcinogens that were classified as initiators and promoters[29]. Following the identification of cellular oncogenes, experiments in tissue culture revealed that activation of a single oncogene is not sufficient for cell transformation and tumour development[30]. Indeed, while the introduction of RAS oncogenes into immortalized rodent cells was able to produce transformed cell foci on a monolayer or in soft agar[31], the same procedure was ineffective when applied to primary cells[30]. Soon the concept of cooperation between oncogenes emerged when it was shown that the MYC and RAS oncogenes are able to produce transformed cell foci when co-expressed in primary rat fibroblasts[30]. The family of RAS proteins comprises three homologous proteins, HRAS, KRAS, and NRAS. RAS proteins serve as transducers of cell surface receptors to intracellular effector pathways. They alternate between an inactive GDP-bound state and an active GTP-bound state. Most mutations occurring in a RAS oncogene will impair its GTP hydrolysis function such that RAS remains in a GTP-bound active state. This leads to sustained activation of downstream effector pathways such as proliferation and differentiation[32,33]. In addition to missense mutations, there is evidence suggesting that RAS mutant overexpression is required for tumour development[34]. Indeed, it was proposed that Ras-induced tumorigenesis involves two steps: the acquisition of an activating mutation and overexpression of the activated Ras allele[34].

The cooperation between MYC and RAS received additional confirmation from experiments in transgenic mice: mice that expressed both MYC and RAS transgenes developed more tumours and with a shorter latency period[35]. The concept of cooperating events was extended with the realization that a RAS oncogene could also collaborate with a dominant-negative mutant of p53 or a viral protein such as Large T or Adenovirus E1A that inactivates a tumour suppressor protein[36-39]. Not only can two oncogenes cooperate, but an oncogene can also cooperate with the inactivation of a tumour suppressor gene. A further conceptual advance in our understanding of cooperating events was realized with the description of stress phenotypes of cancer cells[40]. In essence, the tumorigenic state generates additional pressures that impose a block on cell proliferation and eventually induce cellular senescence or apoptosis. To surmount this block and continue to proliferate, cancer cells require extensive adaptation in cellular processes that are not oncogenic per se. As a result, cancer cells become acutely dependent on the increased activity of some normal proteins. The term "oncogene-addiction" had previously been introduced to illustrate the dependence of cancer cells on a tyrosine kinase oncogene such as BCR-ABL[41,42]. The dependence of cancer cells on non-oncogenic proteins was designated "non-oncogene addiction"[40]. In summary, tumour development involves cooperation between the activation of some oncogenes, the inactivation of some tumour suppressors and the increased expression and/or activity of several normal proteins. A striking case of extensive adaptation is illustrated by the response of cancer cells to oxidative stress and oxidative DNA damage.

OXIDATIVE STRESS IN CANCER CELLS AND CELLULAR SENESCENCE

Among the stress phenotypes of cancer cells, oxidative stress and resulting oxidative DNA damage have emerged as critical players. The study of mouse fibroblasts transformed with a RAS oncogene revealed that these cells produce large amounts of reactive oxygen species (ROS)[43]. In fact, elevated ROS production is not limited to cancer cells that harbour a RAS oncogene but has also been observed in cancer cells that harbour a mutation in another gene of the RAS or the phosphatidylinositol-3 kinase (PI3K) pathway (NF1, PIK3CA, PTEN) or an upstream tyrosine kinase (Met or BCR-ABL)[44-51]. Mechanistic studies identified multiple sources of ROS production in cancer cells, such as increased expression and activity of the enzymes NADPH oxidase 1 and 4 (NOX1, NOX4) and cyclooxygenase-2 (Cox-2), transcriptional repression of sestrin family genes, and increased ROS production by the cytochrome c-oxidoreductase in the mitochondrial electron transport chain[52-59] [Figure 2]. Sestrins play an important role in the regeneration of peroxiredoxins and, as such, contribute to the antioxidant firewall[60]. Importantly, as cell proliferation was inhibited by treatment with a chemical antioxidant or knockdown of genes encoding these respective enzymes, it was concluded that ROS play an important role in mediating the mitogenic effect of RAS[43,52,54-57]. However, it was quickly realized that following an initial period of accelerated proliferation, elevated ROS levels also cause the appearance of cells that exhibit markers of cellular senescence such as $p16^{INK4a}$ expression and β-galactosidase activity at pH 6[61]. The induction of cellular senescence by oncogenes was rapidly confirmed in mouse models[62,63]. Experiments in the animal also introduced an important twist to the unfolding story. Indeed, senescent cells were detected in premalignant tumours but not in malignant ones[62]. For example, $BRAF^{V600E}$ induced benign lung tumours that only rarely progressed to adenocarcinoma[63]. Likewise, in human tissues, senescent cells were not detected in malignant tumours but were observed in pre-cancerous lesions such as colon adenomas[64-66], benign tumours of melanocytes (naevi) caused by the *BRAF*^{V600E} mutation[67], and neurofibromas resulting from Neurofibromatosis (NF1) inactivation[68]. In this context, cellular senescence is deemed a tumour suppression mechanism that functions by eliminating early neoplastic cells from the proliferative pool (reviewed in[69]). It should be noted, however, that the presence of senescent cells in tissues has the potential to stimulate the proliferation of tumour cells through the senescence-associated secretory pathway (reviewed in[69]).

ADAPTATION OF CANCER CELLS TO OXIDATIVE STRESS

Cellular senescence protects us against tumour development by preventing the proliferation of cancer cells that exhibit higher ROS levels. Unfortunately, some rare cancer cells manage to adapt by developing mechanisms to prevent the deleterious effects of excessive ROS levels [Figure 2].

Increased production of antioxidants

One mechanism of adaptation involves the increased production of antioxidants [Figure 2]. Early studies using a proteomic approach documented the upregulation of enzymes involved in cellular redox balance, including catalase, peroxiredoxin 3, thioredoxin peroxidase, and γ-glutamyltransferase 2 (GGT2)[70-72]. Subsequent studies described various mechanisms by which cancer cells maintain the ratio of reduced to oxidized glutathione (GSH/GSSG) and nicotinamide adenine dinucleotide phosphate (NADPH/NADP+), notably by reprogramming the metabolism of glutamine, glucose, and fatty-acid[73-76]. Some important players in the response of cancer cells to elevated ROS levels are the nuclear factor erythroid-2 related factor 2 (NRF2) and its repressor protein Kelch-like ECH-associated protein 1 (KEAP1) [Figure 2]. NRF2 is a redox-sensitive transcription factor that binds to the antioxidant response element (ARE) and activates many genes that code for antioxidants and detoxification proteins[77]. KEAP1 binds to NRF2 and negatively regulates its activity by targeting it to proteasomal degradation[78]. In normal cells, the interaction between KEAP1 and NRF2 is disrupted by oxidants and electrophilic agents, thereby allowing NRF2 to translocate to the nucleus and activate the transcription of antioxidant genes[78]. In many cancer cells, NRF2 has become

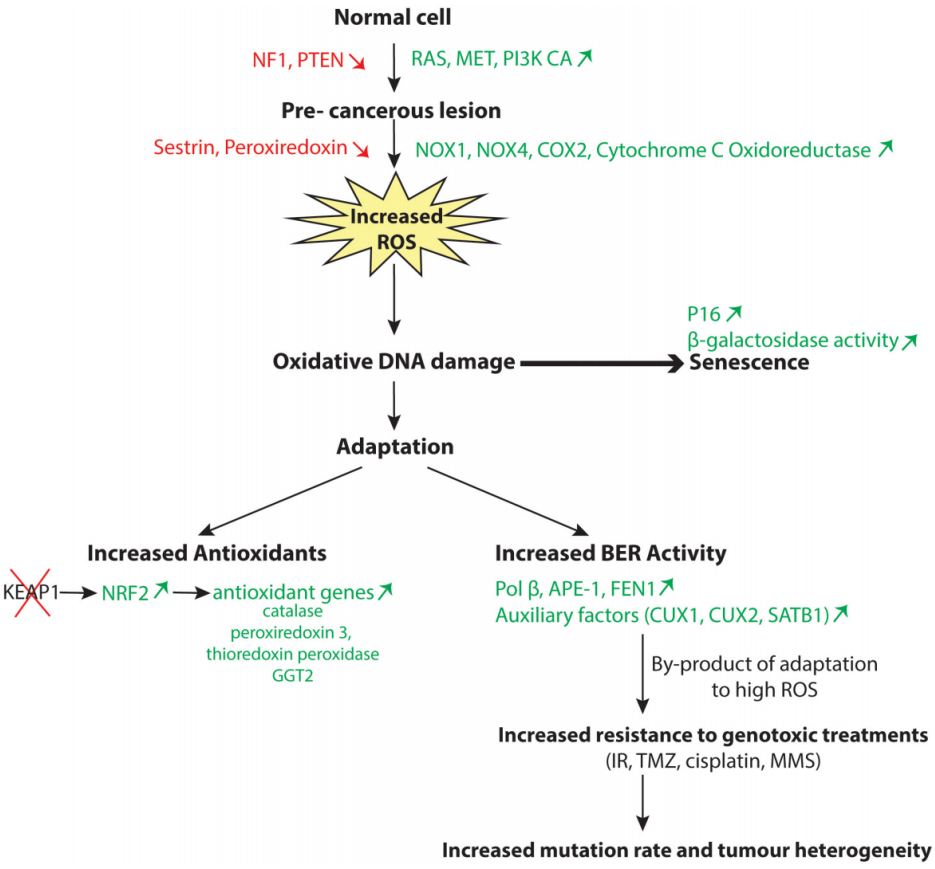

Figure 2. Adaptation of cancer cells to oxidative stress. Activation of the RAS or PI3K pathway leads to elevated production of reactive oxygen species (ROS), which causes oxidative DNA damage and, ultimately, cellular senescence. Two modes of adaptation have been described to enable cancer cells to avoid cellular senescence and continue to proliferate. Some cancer cells increase their antioxidant capabilities notably following genetic inactivation of the Kelch-like ECH-associated protein 1 (*KEAP1)* gene which leads to nuclear factor erythroid-2 related factor 2 (NRF2) upregulation and the subsequent transcriptional activation of antioxidant genes. Alternatively, some cancer cells increase their BER capacities by overexpressing BER enzymes such as Pol β, APE1, and FEN1 or auxiliary factors such as CUX1, CUX2, or SATB1. As a by-product of increased BER activity, these cancer cells exhibit resistance to genotoxic treatments like radiation therapy(IR), temozolomide (TMZ), cisplatin, or methyl methanesulfonate (MMS).

constitutively active following the acquisition of inactivating mutations within the *KEAP1* gene or missense mutations within either the *KEAP1* or *NRF2* gene that weaken the interaction between the two proteins[79-83] (reviewed in[84]). It was estimated that somatic mutations in KEAP1 and NRF2 are present in 15% and 10% of lung cancers, respectively[84]. The existence of mutations that disrupt the KEAP1/NRF2 regulatory system in cancer cells sheds new light on the observed "oncogene-directed" increased expression of NRF2 whereby the expression of KRAS, BRAF, and MYC oncogenes in primary murine cells was found to cause an increase in NRF2 mRNA and protein levels[85]. This and other studies suggested a causal link between RAS and increased NRF2 expression and its transcriptional targets as if a downstream effector of the RAS pathway would act directly to upregulate NRF2[58,70-72,76,85]. Instead of direct induction of NRF2 by the RAS pathway, we envision that cancer cells with higher expression of antioxidants emerge by a Darwinian process of natural selection. Following sustained activation of the RAS pathway, virtually all cells in a population will be negatively affected by excessive ROS levels, but rare cells that express high levels of antioxidants will continue to proliferate and will gradually represent an increasing fraction of the population. This notion is supported by two observations. First, ectopic expression of KRAS or other oncogenes in murine primary cells did not have any effect on NRF2 expression when investigated one week

after the retroviral infection[85]. Increased NRF2 expression was observed only in mouse embryo fibroblasts derived from mice that harbour the KRAS oncogene or another oncogene[85]. Therefore, we suppose that embryonic development up to day 13 allowed enough time for some adaptation. Secondly, if the RAS pathway was able to directly upregulate NRF2 expression, tumour development would not require somatic mutations in *KEAP1* or *NRF2* that abrogate the interaction between the two proteins.

Increased capacity to repair oxidative DNA damage

Several observations concur to indicate that adaptation to elevated ROS production in cancer cells can involve an increase in DNA repair capacity, notably of the BER pathway [Figure 2].

Overexpression of BER enzymes in cancer

Early studies have documented the increased expression and/or activity of BER enzymes in tumour samples. Pol β was first reported to be overexpressed in many cancer cell lines as well as in breast, colon, and prostate adenocarcinomas[86]. The analysis of 68 tumour samples of various tissue of origin estimated that Pol β was overexpressed between 2 to 12-fold in 40% of samples[87]. Pol β expression and activity were significantly higher in blood samples from 9 chronic myelogenous leukemia patients than that from healthy donors[88].

Immunohistochemistry analysis of a panel of ovarian cancers revealed a wide range of APE1 expression with considerable heterogeneity within the same tumour[89]. In prostate tissue, APE1 overexpression correlates with tumour progression: it was detected in 6 out of 100 (6%) benign prostate hypertrophy, 58 out of 100 prostatic intraepithelial neoplasia, and 67 out of 100 (67%) prostate cancers[90]. Measurements of APE1 activity in extracts of 58 glioblastomas and adjacent histologically normal brains revealed increased enzyme activity in 93% of tumour/normal pairs[91]. APE1 activity was also greater in high-grade than in low-grade tumours[91]. APE1 was found to be overexpressed in 43 out of 60 (72%) osteosarcomas, and a significant correlation was observed between elevated APE1 expression and shorter survival[92]. The analysis of 82 cases of oral tongue squamous cell carcinoma showed higher APE1 expression in 53 (64%) samples[93].

The flap structure-specific endonuclease (FEN1) has been reported to be highly expressed in lung cancer cell lines and in multiple types of tumours, including neuroblastomas, prostate, gastric, pancreatic, and non-small cell lung cancers[94-98].

BER enzymes identified in a synthetic lethality screen of RAS-driven cancer cells

A requirement for an efficient BER pathway in RAS-driven cancer cells was first suggested from a genome-wide screen to identify synthetic lethal interactions with the RAS oncogene[99]. The screen was performed using a pair of isogenic colorectal cell lines (DLD-1) that carry or not an endogenous activating $KRAS^{G13D}$ mutation. While stringent statistical criteria ($P \leq 0.1$) identified 368 genes whose knockdown is synthetic lethal to $KRAS^{G13D}$-driven cancer cells, a more relaxed cut off ($P \leq 0.3$) extended this list to 1741 genes, among which were four genes involved in BER: *NEIL2*, *XRCC1*, *POLβ*, *LIG3*[99]. These results indicated that these four BER genes are required for the proliferation of $KRAS^{G13D}$-driven DLD-1 cells to a greater extent than for the control cells. The *CUX1* gene was also found in the list of potential synthetic lethality genes, but the molecular basis for its requirement was not understood until later.

Requirement for CUX1 function as a BER accessory factor in RAS-driven cancer cells

Transgenic mice expressing the p200 CUX1 protein under the control of the mouse mammary tumour virus regulatory sequences developed mammary tumours with a penetrance of ~40% but with a long latency period of 70 to 100 weeks[100]. Such a long latency period indicates that *CUX1* is probably not an oncogene per se, and that, certainly, other cooperating events are required for tumour development in cells that

overexpress CUX1. Interestingly, 44% of tumours from *Cux1* transgenic mice harboured an activating mutation at codon 12 or 61 of *Kras*. Such a high frequency of spontaneous mutations is striking. This implies that the probability that a mouse will acquire an activating mutation in the *Kras* oncogene during its life is approximately 17% (40% × 44%). Yet, wild-type mice do not develop cancer at this rate. The reason for this, fortunately, is that RAS oncogenes trigger a cellular senescence response after an initial period of accelerated proliferation[62-68] (reviewed in[69]). Therefore, other cooperating events are required for pre-cancerous cancer cells to avoid senescence and continue to proliferate. The cooperation of CUX1 with a RAS oncogene in tumour development was confirmed by performing lentiviral infections in the lung of mice. The combination of CUX1 with RAS produced a higher number of cancerous lesions of a larger size than RAS alone. Importantly, while RAS alone caused the appearance of adenomas that exhibited hallmarks of cellular senescence, the combination of CUX1 and RAS produced higher grade adenomas which, in one case, evolved to the adenocarcinoma stage[100]. DNA repair assays *in vitro* with purified proteins established that the p200 CUX1 protein functions as an accessory protein that stimulates the enzymatic activities of several BER enzymes: OGG1, APE1, and Pol β [Figure 1][100-102]. Overexpression of p200 CUX1 accelerates the repair of oxidative DNA damage following treatment with H_2O_2, whereas CUX1 knockdown increases genomic DNA damage, as observed by comet assays and the measurement of abasic sites and 8-oxo-deoxyguanine bases[100]. While the introduction of a RAS oncogene into cells causes an increase in ROS levels that are associated with an increase in DNA damage and the number of senescent cells, simultaneous expression with p200 CUX1 eliminates the increase in genomic DNA damage and senescent cells without affecting ROS elevation[100]. In normal cells, the role of CUX1 as a BER accessory factor is not essential for survival, as demonstrated from lethality screens in human cells and the viability of *Cux1*$^{-/-}$ knockout mice[103-106]. This biochemical activity of CUX1 appears to be needed only in abnormal situations of oxidative stress. For example, mouse embryo fibroblasts (MEFs) from *Cux1*$^{-/-}$ mice proliferate normally in a 3% oxygen atmosphere but senesce immediately when placed at 20% oxygen[107].

Studies in human cancers show that CUX1 is overexpressed in over 70% of cancers. The Cancer Genome Atlas (TCGA) characterization of 276 human colorectal cancers ranked *CUX1* as the fifth gene on a scale showing a correlation between tumor aggressiveness and a combined score based on gene expression and somatic copy number alterations[108]. In glioblastomas, TCGA reveals that the chromosomal region including 7q22, where *CUX1* resides, is the most frequently and highly amplified chromosomal region[109]. TCGA and REMBRANDT data also show shorter survival of glioblastoma patients with high *CUX1* mRNA expression[109] (reviewed in[101]). In smaller-scale studies, immunohistochemical analyses on breast, pancreas, and glioblastoma cancers reveal that CUX1 expression inversely correlates with relapse-free and overall survival[101,110-112].

CUX1 knockdown does not impair the clonogenic efficiency of cancer cell lines that exhibit low ROS levels but is synthetic lethal in all cancer cells that display elevated ROS levels, whether this results from an activating mutation in a *RAS* gene (Hs578T^{HRAS}, MDA-MB-231^{KRAS}, DLD-1^{KRAS}, HCT116^{KRAS}, KE37^{NRAS}), another gene in the pathway (HT29^{BRAF}), or an upstream receptor tyrosine kinase (HCC827^{EGFR})[100,113]. Strikingly, CUX1 knockdown does not affect the viability of A549 lung carcinoma cells which harbour an activating KRAS^{G12D} mutation[113]. The reason for this discrepancy is that these cells also carry an inactivating mutation in the KEAP1 tumour suppressor gene[82,83]. As a result, the NRF2 transcription factor accumulates in the nucleus of A549 cells and activates an antioxidant transcriptional program[82].

Other CUT domain proteins as BER accessory factors

Structure-function analysis identified the CUT domains within CUX1 as the protein region responsible for the stimulation of OGG1, APE1, and Pol β *in vitro*[100-102,107]. In cells, a protein containing the CUT domains 1

and 2, together with a nuclear localization signal, was sufficient for recruitment to laser-induced DNA damage[113]. In cancer cells that exhibit high ROS levels, the CUT domains 1 and 2 reduced genomic DNA damage as measured by comet assay at pH 13, and comet assay at pH 10 after treatment with the Fapy DNA glycosylase (FPG)[100-102]. Moreover, the CUT domains 1 and 2 conferred resistance to multiple genotoxic agents, including H_2O_2, ionizing radiation, temozolomide, MMS, and cisplatin[100-102,107,113]. In addition, the CUT domains 1 and 2 can restore the capacity of *Cux1*$^{-/-}$ MEFs to proliferate in 20% oxygen[107].

The CUT domains are evolutionarily conserved domains that are present in a few transcription factors in humans [Figure 3]. While CUX1 is ubiquitously expressed, CUX2 is mainly expressed in the nervous system[114-116] and can also function as a female-specific transcription factor in the liver[117]. SATB1 is present in specific cell types such as thymocytes and basal layer cells of the epidermis where it regulates the expression of a large set of genes by organizing specific chromosome loci into small chromatin loops[118-121]. SATB1 has been implicated in various cancers such as breast cancers, cutaneous malignant melanoma, gastric and colorectal cancers[122-125].

Not surprisingly, these other CUT domain proteins were found to function as BER accessory factors [Figure 1]. Knockdown of CUX2 or SATB1 was found to cause a delay in the repair of oxidative DNA damage and an increase in oxidized purines in genomic DNA, whereas ectopic expression of these proteins accelerated DNA repair and reduced the amount of genomic DNA lesions[126,127]. As for CUX1, enzymatic assays with purified proteins mapped the DNA repair activity of CUX2 and SATB1 to the CUT domains[126,127]. Strikingly, both CUX2 and SATB1 are overexpressed in certain cancer cells where their knockdown increases genomic DNA damage and impairs the capacity of cancer cells to proliferate[126,127]. The case of CUX2 is particularly remarkable. CUX2 is not expressed in the mammary gland. Yet, a genome-wide shRNA screen revealed that CUX2 is required for proliferation in several breast cancer cell lines[128]. The aberrant overexpression of CUX2 in breast cancer cells suggests that elevated ROS levels in cancer cells can select rare cells that express any protein able to accelerate the repair of oxidative DNA damage.

The mechanism by which CUT domains stimulate the enzymatic activity of BER enzymes is not always clear. In the case of OGG1, electrophoretic mobility shift assays (EMSA) established that CUT domains stimulate the binding of OGG1 to DNA that contains oxidized purines[107]. On the one hand, CUT domains can interact directly with OGG1[107,126,127]. On the other hand, in EMSA, CUT domains were observed to exhibit affinity to DNA that contains an oxidized purine (Ramdzan and Nepveu, unpublished observation). CUT domains were also shown to interact directly with APE1 and Pol β[101,102]. However, whether CUT domains accelerate the binding of these enzymes to their DNA substrates remains to be verified.

Other transcription factors that function as BER accessory factors

Other transcription factors and DNA binding proteins were reported to play a role in BER. YB-1 and hnRNP-U were shown to stimulate NEIL1[129,130]. HMGB1 was reported to stimulate the functions of the APE1 and FEN1 endonucleases[131]. The FACT complex was also shown to facilitate uracil removal by UDG[132]. *In vitro,* P53 stimulates the activity of Pol β[133]. These findings suggest that there may be a wide variety of accessory proteins acting to facilitate BER.

CONSEQUENCES OF INCREASED BER ACTIVITY IN CANCER CELLS

Apart from being able to avoid senescence and continue to proliferate, the increased DNA repair capability of cancer cells that overexpress BER enzymes and accessory factors confers additional properties that have a major impact on resistance to treatment, tumour progression, and ultimately, patient survival.

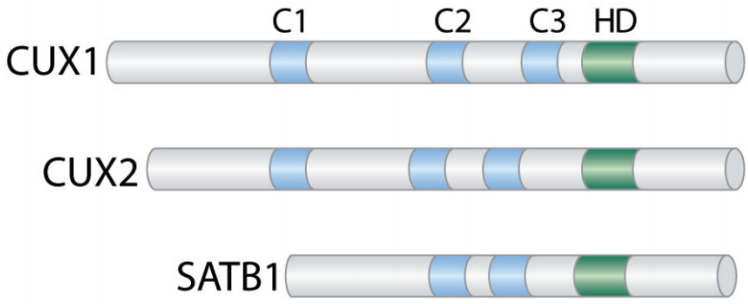

Figure 3. CUT Domain Proteins. Diagrammatic representation of the CUT domain proteins CUX1, CUX2, and SATB1. The evolutionarily conserved CUT domains (C) and Cut homeodomain (HD) are shown.

Impact of BER enzymes on resistance to treatments

There is ample evidence to demonstrate that resistance to genotoxic treatments correlates with expression and activity of several BER enzymes, notably Pol β, APE1, and FEN1 (reviewed in[134]). High Pol β expression confers resistance to genotoxic treatments[135-139]. Conversely, Pol β knockdown or inhibition sensitizes cancer cells to various treatments including temozolomide[137,139-142], MMS[143-146], oxaliplatin[147], cisplatin and UV-radiation[148]. Elevated APE1 expression in cancer cells has been associated with poor response to mono-alkylating agents[91,149-151], whereas various approaches to reduce APE1 activity were shown to increase cancer cell sensitivity to temozolomide[149,152-158]. Knockdown or inactivation of the FEN1 gene sensitized cells to gamma-radiation, MMS, temozolomide, and cisplatin[159,160]. The N-methylpurine DNA glycosylase (MPG) was also shown to promote resistance to mono-alkylating agents[161,162]. However, results also emphasized the importance of balancing glycosylase activity with that of Pol β[137,139]. Indeed, unless repair of the base lesion is brought to completion, removal of alkylated bases produces abasic sites and single-strand breaks that can be more toxic than the original lesions.

Impact of BER accessory factors on resistance to treatment

As a by-product of their adaptation to high ROS levels, cancer cells that have increased their capacity to repair oxidative DNA damage through elevated expression of BER accessory factors also exhibit increased resistance to genotoxic treatments. Here, we will describe the documented effects of BER accessory factors on resistance to treatment.

There exists old literature linking RAS oncogenes with radioresistance[163-166]. We consider it likely that the higher resistance to ionizing radiation of some RAS-driven cancer cells is a by-product of their adaptation to elevated ROS levels through the enhanced expression of BER enzymes and accessory factors. We previously mentioned that *CUX1* knockdown did not affect the viability of cancer cell lines with low ROS levels. Strikingly, however, knockdown of *CUX1* or *SATB1* sensitized all tested cancer cell lines to ionizing radiation, whether they displayed high ROS levels or not[101,113,127]. In turn, ectopic expression of CUX1 or the small recombinant protein containing only two CUT domains increased the resistance to radiation[101,113]. In glioblastoma cells, resistance to the mono-alkylating agent temozolomide was reduced by CUX1 knockdown but increased by ectopic expression of CUX1 or the two CUT domains[101]. Similar results were obtained following treatment with another alkylating agent, MMS[101]. The standard-of-care treatment for glioblastoma patients involves a combined treatment with ionizing radiation and temozolomide. Resistance to combined treatment was reduced by *CUX1* knockdown but increased by overexpression of CUX1 or the two CUT domains[101]. Moreover, the resistance of cancer cells to cisplatin treatment was decreased by *CUX1* knockdown but increased by ectopic expression of the two CUT domains[102]. These findings are in line with the results of *in vitro* assays showing that the CUT domains stimulate the cleavage activity of APE1 as well as the deoxyribose phosphate lyase and the polymerase activities of Pol β, and the bypass of

intrastrand G-crosslink by Pol β[101,102].

Contribution of elevated ROS levels and BER activity to acquired resistance and tumour progression

We previously described that the ~30,000 endogenous, daily, damaged bases in diploid human cells combined with a Pol β error rate of at least 1/1000 contributes to the intrinsic mutation process that makes our somatic cells accumulate mutations through life. This process is exacerbated in cancer cells in which increased BER activity enables them to survive despite elevated ROS levels. These cells suffer a much higher number of oxidative DNA lesions than normal cells; and assuming a similar error rate by Pol β, it is reasonable to assume that these cells exhibit a mutator phenotype and acquire point mutations at a faster rate. This notion received confirmation from a study of acquired resistance to imatinib by chronic myelogenous leukemia (CML) cells[49]. The authors showed that the BCR-ABL kinase causes an increase in ROS levels and oxidative DNA damage that is associated with a rise in mutation rates as measured by resistance to ouabain or imatinib[49]. These effects were reduced by treatment with antioxidants or expression of a BCR-ABL^{Y177F} mutant that does not elevate ROS[49]. ROS production induced by BCR-ABL was subsequently shown to increase chromosomal aberrations[167].

The acquisition of resistance to imatinib by some BCR-ABL CML cells illustrates another consequence of elevated ROS levels and BER activity. The higher rate of mutation in these cancer cells contributes to tumour heterogeneity, a feature that constitutes a major obstacle to successful clinical treatments. Although a specific treatment can manage to kill more than 99.99% of cancer cells, if only one rare cancer cell acquires a mutation that allows its survival, selective pressure will promote the expansion of this cell clone. At the clinical level, this leads to cancer relapse.

CONCLUDING REMARKS

Evidence from *in vitro* assays with purified proteins, tissue culture cells, and transgenic mice reveals that the efficiency of BER in mammalian cells can be modulated by the action of auxiliary factors, notably the CUT domain proteins. Although these accessory factors are not essential, they appear to protect cells against oxidative DNA damage in situations of oxidative stress. This biochemical activity is hijacked by cancer cells to avoid senescence and continue to proliferate in the presence of excessive ROS levels [Figure 2]. As a by-product of this mechanism of adaptation to elevated ROS levels, cancer cells with enhanced BER activity exhibit increased resistance to genotoxic treatments and a higher mutation rate that contributes to tumour heterogeneity. However, the acute dependence of some cancer cells on DNA repair accessory factors may have uncovered an Achilles' heel that could be exploited in future therapeutic strategies. In the past, the realization that BER enzymes contribute to therapy resistance led to many drugs that inhibit BER enzymes being tested in the clinic with various treatment modalities. The drawback of such approaches is that BER enzymes are essential to normal cell viability, since over 30,000 base alterations per day are produced endogenously in a normal human cell[12]. Consequently, although inhibitors of BER enzymes increase cell killing within the tumor, they also cause severe adverse effects that considerably reduce the therapeutic window. As DNA repair accessory factors are not essential to normal cells in regular physiological situations, specific inhibitors to these proteins could be deleterious to cancer cells without causing many adverse effects, thereby increasing the therapeutic window.

DECLARATIONS

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Authors' contributions

Write the manuscript and prepare figures: Vickridge E, Faraco CCF, Nepveu A

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Cancer Drug Resistance

Perspective

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GD2+ cancer stem cells in triple-negative breast cancer: mechanisms of resistance to breast cancer therapies

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Abstract

Research has led to the development of tailored treatment options for different cancers in different patients. Despite some treatments being able to provide remarkable responses, nearly all current treatments encounter the same issue: resistance. Here, we discuss our experiences with how breast cancers resist therapies. The focus of our discussion revolves around the cancer stem cell subpopulation and their mechanisms for resistance.

Keywords: Drug resistance, breast cancer therapy, triple-negative breast cancer, cancer stem cells

INTRODUCTION

In 2021, breast cancer became the most common globally diagnosed cancer, accounting for 12% of all annual cases, with over 40,000 American women projected to die of breast cancer in 2022[1]. Despite advancing therapies, treatment resistance has been shown to be responsible for up to 90% of cancer-related deaths[2,3]. The purpose of this perspective is to share our group's cumulative experiences in researching breast cancer and the mechanisms of treatment resistance. To do this, we discuss literature and findings from our group and others. The discussions focus on the triple-negative breast cancer (TNBC) subtype and

the cancer stem cell (CSC) subpopulation. Furthermore, we will place emphasis on the CSC biomarker GD2 due to its novelty and integral role in our research. We hope that our text will promote discussions and encourage other research groups to incorporate our understanding and findings into their projects.

Breast cancer subtypes: TNBC and hormone receptor therapies

Breast cancer is a complex malignancy that can be molecularly characterized into subtypes with unique disease progressions, treatment regimens, and prognoses[4]. Subtyping is based on the tumor's hormone receptor status and can be grouped as follows: Luminal A (ER+, PR+, HER2-, KI67-), Luminal B (ER+, PR+, HER2±, KI67+), HER2 overexpression (ER-, PR-, HER2+), and Basal/Triple Negative (ER-, PR-, HER2-)[5]. Although TNBC can be further classified (basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor), the overall characteristics are a highly invasive, metastatic, and recurrent breast cancer subtype with high mortality rates and few treatment options[6,7]. TNBC's lack of receptor expression allows it to resist current hormone receptor therapies such as tamoxifen, aromatase inhibitors, luteinizing hormone-releasing hormone agonists, Faslodex, and trastuzumab[8-10]. As a result, current treatments are limited to conventional therapeutics such as tumor resection, chemotherapy, and radiation therapies[11]. Despite initial responses, TNBC patients have a higher rate of distant recurrence with significantly worse 3-year survival probabilities post-neoadjuvant treatment compared to other breast cancer subtypes[12]. Therefore, identifying specific therapeutic targets for TNBC is imperative for improving treatment outcomes for patients.

Cancer stem cells: resisting chemo- and radiotherapies

Carcinogenesis, the transformation of healthy to cancerous cells, is due to an accumulation of mutations that results in uncontrolled replication and can often begin with a single cell. Despite their monoclonal origin, tumors are composed of a heterogeneous population of cells with only a small subpopulation possessing tumorigenic potential[13]. CSCs are a sub-subpopulation of tumors that possess the ability to self-renew, have increased tumorigenic potential, and increased mobility. Additionally, CSCs are innately resistant to radiotherapy and chemotherapies[14-16]. Although chemo- and radiotherapies may be initially successful in killing off most cells in a tumor, they confer a selection pressure for CSCs. This leads to the recurrence of a treatment-resistant tumor and an overall poorer outcome for TNBC patients in comparison to other breast cancer subtypes.

Chemotherapies and radiotherapy primarily affect rapidly dividing cells by interfering with DNA processing, leading to cellular apoptosis. Cancer stem cells have several mechanisms that allow them to resist and overcome the effects of chemotherapy. For example, CSCs have the ability to become quiescent in stressful conditions, hibernating in G_0 and re-entering the cell cycle once conditions have improved. Malladi *et al.* identified SOX2 and SOX9 transcription factors producing DKK1 to inhibit WNT as the mechanism for quiescence in early-stage human lung and breast carcinoma cell lines[17]. This lack of cell division and, by association, DNA replication decreases the effectiveness of DNA targeting therapies such as chemo- and radiotherapy. In addition to becoming quiescent, CSCs can express ABC transporter proteins and efflux pumps that actively remove toxic compounds from within the cell. Das *et al.* were able to enhance the chemosensitivity of breast cancer stem cells by downregulating ABCG2 using nanoparticles[18]. Similarly, Sun *et al.* enhanced doxorubicin sensitivity, demonstrated by a 90% decrease in IC50, after 60% downregulation of ABCG2 was achieved by siRNA[19]. Although these studies demonstrate the significance of ABC transporters and efflux pumps in CSCs, it is important to note that varying levels of these proteins can be expressed in the non-stem population of the tumor as well.

Apoptosis is the natural cell death response to DNA damage. If quiescence and ABC transport proteins fail to prevent DNA damage from chemo- and radiotherapies, cancer cells will undergo apoptosis. This can be seen during the initial responsiveness of tumors to chemo- and radiotherapies. However, cancer stem cells have intrinsic mechanisms that allow them to avoid apoptosis. Takeda *et al.* identified a ROCK-survivin anti-apoptotic axis in pancreatic cancer stem cells and that inhibiting ROCK resulted in decreased survivin expression, leading to the sensitization of cells to gemcitabine[20]. Although there are more pathways associated with cancer stem cells and their resistance to therapies, such as Hippo/YAP1, Wnt/ß-catenin, Notch, and JAK/STAT, the main takeaway is that cancer stem cells can resist therapies through quiescence, drug efflux, and anti-apoptosis[21]. Overall, these mechanisms make CSCs difficult to target. When combined with hormone receptor therapy resistance, these qualities make TNBC an especially difficult disease to treat. Consequently, understanding the cellular biology of CSCs in TNBC, and other cancers, can provide insight into future novel therapies[22,23].

GD2: a cancer stem cell biomarker

Stem cells are essential for their abilities to self-renew and differentiate and are responsible for migrating to biological niches to replace damaged or depleted cell types[24]. In cancer, these same qualities lead to disease progression, metastasis, and patient mortality. It is important, then, to characterize CSC-specific cell surface markers for targeted treatment. In 2003, Al-Hajj *et al.* identified CD44high/CD24low as breast CSC markers[25]. Shortly after, ALDH1 was identified as another breast CSC marker, although primarily for luminal subtypes[26]. However, these markers are poor targets for therapy because they are also expressed by normal human breast cells[27].

In 2012, Battula *et al.* identified GD2 as a novel breast cancer stem cell marker in TNBC and that it is co-expressed with $CD44^{high}/CD24^{low}$ cells[28]. GD2, a b-series ganglioside located within lipid rafts on the outer side of the plasma membrane, is expressed during development but is highly restricted to cerebellar tissues and peripheral nerves in mature adults. It can also be found on tumors of neuroectodermal origin such as neuroblastoma and melanoma[29,30]. Battula *et al.* and Jaggupilli *et al.* then went on to identify NF-kB signaling, an inflammatory pathway, and metabolic stress, respectively, as primary drivers of GD2 expression[31,32]. Further studies by Nguyen *et al.* identified GD2 as an activator of the FAK-AKT-mTOR signaling pathway, leading to increased tumor growth and metastasis in *in vivo* conditions[33]. Furthermore, this study demonstrated that CRISPR knock-out of GD3S, the rate-limiting enzyme for GD2 synthesis, completely ablated GD2 cells. Interestingly, GD3S KO cells resulted in no *in vivo* tumor growth and metastasis, greatly reduced *in vitro* migration, invasion, and growth in 3D conditions, but had no effect on *in vitro* 2D proliferation. Patient studies have shown that high levels of GD2 have been associated with advanced cancer stage, larger tumor size, nodal invasion, and enhanced tumor proliferation and invasiveness[34]. These clinical correlations make GD2 an ideal breast CSC therapeutic target.

Targeting GD2 to treat cancer stem cells

Characterized by its lack of hormone receptor status, patients diagnosed with TNBC are typically unresponsive to target-specific hormone receptor therapies. Treatment options primarily consist of surgery, neoadjuvant therapies, and chemotherapy with standard cytotoxic agents. Despite initial responses, the overall prognosis remains poor[35,36]. GD2 has been identified as a suitable therapeutic biomarker for targeting breast CSCs because of its limited expression in normal, healthy tissues. Because of this, therapies that target GD2 could be a promising avenue for treatment development. Battula *et al.* identified BMS-345541 as a potential upstream small molecule inhibitor for GD2 by blocking IKK activity, thus suppressing NF-kB signaling[31]. Although NF-kB is ubiquitous in normal tissues and cells, it is typically inactive until cellular stress responses such as inflammation occur. Furthermore, Jaggupilli *et al.* were able to reduce the GD2+ CSC population by 70%-80% using glutamine uptake inhibitor, V9302[32]. Lastly, Nguyen *et al.* were

able to suppress the activity of GD2+ CSCs by inhibiting downstream activation of FAK and mTOR using PF-573228 and everolimus, respectively[33].

In addition to targeting the upstream and downstream mechanisms of GD2 expression and activity, immune-based therapies that target GD2 expressing cells could also be pursued. Seitz *et al.* accomplished this by developing CAR T-cells (chimeric antigen receptor T-cells) that are genetically engineered to target GD2 expressing cells[37]. Although their studies demonstrated anti-GD2 CAR T-cells to be effective at reducing metastasis *in vivo*, graft-versus-host disease (GVHD) is a major cause of clinical morbidity and mortality[38]. To overcome the limitations associated with T-cells, natural killer (NK) cells could be generated instead. Esser *et al.* demonstrated that engineered GD2-specific NK cells were able to induce ADCC-like (antibody-dependent cellular cytotoxicity) killing effects against neuroectodermal tumors[39]. Furthermore, Ly *et al.* determined that infusion of anti-GD2 antibody dinutuximab, a chimeric human-mouse monoclonal antibody, could inhibit tumor growth *in vivo* and extend the survival of mice with TNBC via NK cell-mediated antibody-dependent cellular cytotoxicity[34]. The FDA has already approved GD2-specific therapies for the treatment of neuroblastoma, osteosarcoma, and other GD2+ cancers. Additionally, other anti-GD2 therapies, such as naxitamab and an anti-GD2-GD3 vaccine, have been granted FDA approval[40,41]. Anti-GD2 antibodies, however, do confer toxicity and are associated with adverse side effects including neuropathy, fever, significant pain, and allergies[42]. Neuropathic pain is likely due to GD2 expression in neural cells; however, *O*-acetyl-GD2 (OAcGD2), a GD2 derivative, is expressed on GD2+ solid tumor cells but not on neural fibers, and specific targeting OAcGD2 may reduce the aforementioned side effects[43]. Overall, these compounds may be beneficial for the treatment of TNBC, in combination with other therapies, by targeting the CSC subpopulation.

SUMMARY

Breast cancer is now the most commonly diagnosed cancer, accounting for 12% of global cases. Although advances in breast cancer treatment have been made, treatment resistance remains a problem for many patients. This is especially true for patients diagnosed with triple-negative breast cancer because of this cancer subtype's inherent ability to resist hormone receptor targeted therapies. Additionally, treatment with chemo- and radiotherapies is selected for the cancer stem cell subpopulation, a subset of cancer cells that is resistant due to mechanisms involving quiescence, DNA replication and repair, and apoptosis. Furthermore, these CSCs have an increased tumorigenic and metastatic potential.

GD2 has been identified as a breast cancer stem cell marker and is a promising target for breast cancer therapy[44]. Furthermore, dinutuximab is a GD2-specific monoclonal antibody that is FDA approved for neuroblastoma. Because of this, we believe incorporating methods for targeting CSCs, specifically by GD2, into current TNBC research may lead to promising results. Examples of this would be GD2 combination therapies with novel pathway inhibitors, analyzing the effects in the tumor microenvironment, and studying the role of CSCs on the tumor architecture by looking at the extracellular matrix. Any discoveries could potentially lead to rapid clinical turnarounds due to the FDA-approved status of dinutuximab.

DECLARATIONS

Authors' contributions

Contributed to the conception and writing of this manuscript: Nguyen K
Contributed to reviewing the latest literature in the field, writing, and generation of the graphical abstract: McConnell E, Edwards O
Contributed their experience and gave final approval for submission: Collins-Burow BM, Burow ME

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
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Cancer Drug Resistance

Review

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Liquid biopsies to predict CDK4/6 inhibitor efficacy and resistance in breast cancer

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Abstract

Cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors combined with endocrine therapy have transformed the treatment of estrogen receptor-positive (ER+) and human epidermal growth factor receptor 2 negative (HER2-) metastatic breast cancer. However, some patients do not respond to this treatment, and patients inevitably develop resistance, such that novel biomarkers are needed to predict primary resistance, monitor treatment response for acquired resistance, and personalize treatment strategies. Circumventing the spatial and temporal limitations of tissue biopsy, newly developed liquid biopsy approaches have the potential to uncover biomarkers that can predict CDK4/6 inhibitor efficacy and resistance in breast cancer patients through a simple blood test. Studies on circulating tumor DNA (ctDNA)-based liquid biopsy biomarkers of CDK4/6 inhibitor resistance have focused primarily on genomic alterations and have failed thus far to identify clear and clinically validated predictive biomarkers, but emerging epigenetic ctDNA methodologies hold promise for further discovery. The present review outlines recent advances and future directions in ctDNA-based biomarkers of CDK4/6 inhibitor treatment response.

Keywords: Breast cancer, liquid biopsy, circulating tumor DNA, cell-free DNA, CDK4/6 inhibitors, resistance mechanisms, predictive biomarkers, circulating biomarkers

INTRODUCTION

Breast cancer is the most diagnosed cancer in women globally, with approximately 70% of diagnoses being tumors that express estrogen receptors (ER+) but not human epidermal growth factor receptor 2 (HER2-)[1]. In jurisdictions that have implemented breast cancer screening programs, many ER+/HER2- breast cancers are diagnosed in localized or locoregional stages and are amenable to curative intent therapy. However, despite multimodality treatments, patients have a lifelong risk of metastatic recurrence, and once distant metastasis presents clinically, it is typically incurable[2,3].

In the past decade, novel therapeutic strategies for metastatic breast cancer patients have been implemented in the clinic. Among these, the new standard treatment for ER+/HER2- locally advanced and metastatic breast cancer consists of cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors combined with endocrine therapy[4]. Three CDK4/6 inhibitors have been approved (i.e., abemaciclib, palbociclib, and ribociclib) after displaying significant clinical benefit in pivotal phase III clinical trials[5-12]. CDK4/6 inhibitors have been shown to improve response rate, median progression-free survival (PFS), health-related quality of life, and overall survival (OS) of metastatic breast cancer patients[13-19]. However, CDK4/6 inhibitor resistance remains a significant obstacle. A minority of patients have intrinsic resistance, defined as progression (without response) within six months of starting treatment. Even for patients who experience initial response and clinical benefit from these agents, acquired resistance inevitably develops over subsequent months (median PFS ranges from 23.8-28.2 months in the first-line metastatic setting)[11,17,20]. Therefore, biomarkers are urgently needed to predict CDK4/6 inhibitor efficacy or resistance in metastatic breast cancer patients, allowing clinicians to tailor treatment and potentially add additional therapies for patients at high risk of early progression.

Biomarkers for CDK4/6 inhibitors have been thoroughly investigated through molecular profiling of tumor material, but to date, the only clinically available biomarker remains breast cancer subtype as defined by traditional tissue markers (i.e., ER+/HER2-)[21,22]. Despite significant research efforts, tumor heterogeneity and difficulties distinguishing endocrine resistance from CDK4/6 inhibitor resistance have impeded predictive biomarker discovery[23]. Given practical challenges to obtaining and repeating metastatic tissue biopsies, blood-based profiling of tumor-derived material (i.e., "liquid biopsy") has significant potential to facilitate biomarker explorations. For instance, circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are promising liquid biopsy analytes because they harbor cancer-specific molecular aberrations[24].

In this review, we discuss biomarker-directed treatment for breast cancer, mechanisms of resistance to CDK4/6 inhibitors, and attempts to uncover ctDNA liquid biopsy biomarkers of efficacy and resistance to CDK4/6 inhibitors. Lastly, we highlight undeveloped areas for future advances, namely epigenetic-based liquid biopsy biomarkers for patients treated with CDK4/6 inhibitors.

BIOMARKER-DIRECTED PRECISION ONCOLOGY AND BREAST CANCER

Precision oncology relies on molecular information from each patient's cancer to optimize and individualize treatment regimens. Leveraging these patient-specific molecular biomarkers allows clinicians to select the best treatment for individual patients to improve therapeutic efficacy and reduce adverse effects on healthy cells[25]. Molecular biomarkers include prognostic biomarkers, which may provide information on the expected disease course independent of treatment, and predictive biomarkers, which provide insight into the effect of a specific therapy. Both prognostic and predictive biomarkers may be used to personalize treatment via risk-stratification or directing effective treatments.

Current standard-of-care breast cancer treatments provide several archetypal examples of biomarker-directed precision oncology. For instance, OncotypeDx is a commercial 21-gene assay for ER+ early-stage breast cancer that returns a recurrence score indicating the probability of relapse without adjuvant chemotherapy, with higher scores associated with a poorer prognosis. OncotypeDx serves a prognostic role; its clinical utility stems from identifying patients with a higher absolute recurrence risk and, therefore, a higher likelihood of benefiting from adjuvant chemotherapy[26,27]. Similarly, the MammaPrint microarray assay is a prognostic biomarker that uses the expression levels of 70 genes to classify patients according to recurrence risk[28,29].

Other breast cancer biomarkers highlight the impact of predictive biomarkers in precision oncology. Intrinsic molecular subtypes and associated hormone receptors (ER, PR) and HER2 expression levels are critical for drug selection in breast cancer patients[30]. For instance, HER2+ breast cancer preferentially responds to HER2-targeted agents, such as trastuzumab and trastuzumab emtansine (T-DM1)[31]. Likewise, hormone receptor expression denotes tumors that preferentially respond to endocrine therapy. Resistance to endocrine therapy can occur over time through a variety of mechanisms (e.g., genetic alterations of *ESR1*, increased activity of cyclin-dependent kinases (CDKs), and mitogen-signaling pathways such as PI3K and RAS, or a decrease in proteins that inhibit CDKs such as p16, p21, and p27), several of which converge on the cyclin D-CDK4/6 axis[32]. Therefore, simultaneous treatment with endocrine therapy and CDK4/6 inhibitors has emerged as a highly successful treatment paradigm for ER+/HER2- metastatic breast cancer.

MOLECULAR MECHANISMS OF CDK4/6 INHIBITOR EFFICACY AND RESISTANCE

The cyclin D-CDK4/6-Retinoblastoma protein (Rb) axis regulates cell cycle progression from G1 to the S phase [Figure 1]. Before entering the cell cycle, Rb is hypophosphorylated and bound to the E2F transcription factors (TFs), causing their inhibition[33]. When the appropriate mitogenic signals are present, quiescent cells may enter the cell cycle at the G1 phase. These mitogenic signals lead to the expression of cyclin D, which competes with CDKN2 family proteins to bind CDK4/6, forming the cyclin D-CDK4/6 complex[34]. This active complex can then phosphorylate Rb, causing a conformational change and subsequent release of the E2F TFs, which drive S phase entry and further cell cycle progression via downstream transcriptional activation[33]. Furthermore, the cyclin D-CDK4/6 complex triggers the forkhead box protein M1 (FOXM1) TF, promoting the advancement of later cell cycle phases (G2/M)[35]. ER+ breast cancer is highly reliant on an intact cyclin D-CDK4/6-Rb axis, as estrogen drives cyclin D1 expression leading to the formation of the cyclin D-CDK4/6 complex, ultimately inducing cell proliferation through the CDK4/6 pathway[36]. CDK4/6 inhibitors leverage this by binding the ATP domain of CDK4/6 and halting progression from the G1 to S phase of the cell cycle[37].

The mechanisms of resistance to CDK4/6 inhibitors have yet to be fully elucidated, and in many cases, the clinical relevance of putative mechanisms discovered in preclinical models remains unconfirmed[38,39]. Recognized resistance mechanisms include amplification of members of the cyclin D-CDK4/6 axis or downregulation of CDK4/6 repressor proteins, such as p21 and p27, which may thwart the direct effects of these drugs[38,40-42]. Additionally, alterations in *RB1*, *FAT1*, or the PI3K/AKT/mTOR and KRAS signaling pathways may act to circumvent the G1/S checkpoint in the presence of CDK4/6 inhibitors, inducing cell cycle progression independent of the cyclin D-CDK4/6 axis[38,39,43-46].

CIRCULATING CELL-FREE DNA FOR LIQUID BIOPSY AND COMPANION DIAGNOSTICS

In recent years, liquid biopsy approaches have emerged, intending to provide molecular information for biomarker-directed precision oncology from a fluid sample[24]. Traditionally, accessing this information has required invasive procedures, like tissue biopsy, which are not always feasible depending on the nature and

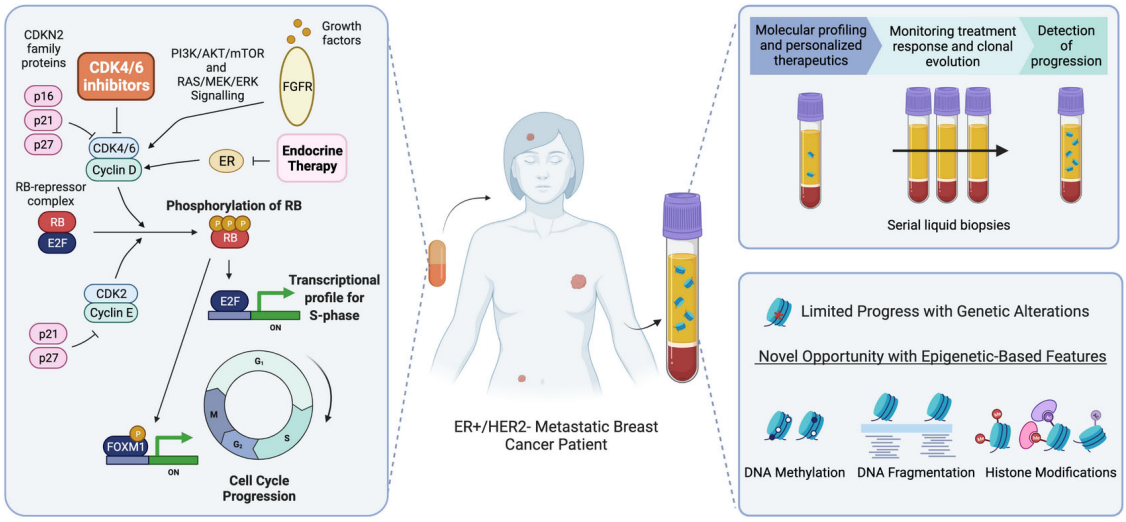

Figure 1. Overview of CDK4/6 inhibitor treatment and liquid biopsy in ER+/HER2- metastatic breast cancer patients. CDK4/6 inhibitors prevent the phosphorylation of Rb and downstream activation of the transcriptional profile required for progression to the S-phase of the cell cycle. In the anti-CDK4/6 therapy clinical setting, a liquid biopsy may be used to direct therapy, monitor patient response over time, and detect progression. Currently, research efforts focused on monitoring genetic alterations in cfDNA through liquid biopsy have made limited progress. Epigenetic profiling of cfDNA may reveal new biomarkers of CDK4/6 inhibitor efficacy or resistance.

location of the tumor, as well as the health of the patient. These limitations are particularly relevant for metastatic breast cancer patients, where ER+/HER2- breast cancer recurrences often occur past 5 years. In this clinical setting, the primary tissue sample may not represent metastatic disease, and the metastatic sites are often in areas difficult to biopsy (e.g., bone and lung)[47,48]. Here, a liquid biopsy may bypass the procedural, spatial, and temporal obstacles of tissue biopsy by encompassing disease heterogeneity and new metastases while being easily repeated throughout tumor progression and different lines of treatment[49]. Therefore, liquid biopsy is advancing as a valuable approach to enable contemporaneous and minimally invasive testing of tumor-specific analytes in cancer patients[50].

For most liquid biopsy applications, the body fluid of choice is peripheral blood plasma. This has previously presented challenges in detecting brain tumors or metastatic sites, likely due to the blood-brain barrier; however, newer liquid biopsy technologies have led to improvements in sensitivity[51,52]. Regardless, other accessible fluids may be more informative depending on the tumor type (e.g., saliva for oral cancer, urine for bladder cancer, and cerebrospinal fluid for glioma)[53]. There are also a variety of components from the tumor which may be assessed through liquid biopsy, including intact tumor cells (e.g., CTCs), cell-free DNA (cfDNA), extracellular vesicles, cell-free RNA, and more[24]. Herein, we focus our discussion on ctDNA as a liquid biopsy biomarker of response to anti-CDK4/6 therapy due to the wealth of recent studies on this topic and the precedent of ctDNA in other settings of precision oncology (e.g., for EGFR tyrosine kinase inhibitors in lung cancer)[49,54]. Other liquid biopsy analytes and their role as biomarkers for CDK4/6 inhibitor treatment have been reviewed elsewhere[23,55,56].

ctDNA molecules containing tumor-derived genetic and epigenetic features are released into the bloodstream as tumor cells die[49]. ctDNA usually represents a small portion of the total circulating cfDNA pool, where most other cfDNA molecules are derived from cells of hematopoietic origin and alternative tissues[57-59]. The release of ctDNA is related to cellular turnover (i.e., many apoptotic and necrotic cells), during which a subset of double-stranded DNA fragments associated with chromatin components enters

the extracellular space[60-62]. ctDNA levels can be as low as < 0.01% of the entire cfDNA pool, with variations depending on tumor size, stage, anatomical location, treatment, and the biological propensity of tumor cells to release their DNA into the circulation[51,58,63-65]. Once in the circulation, clearance of ctDNA by multiple mechanisms (e.g., nuclease-mediated degradation, phagocytosis, and renal excretion) occurs quickly with a half-life between 16 minutes and two hours[66-68]. This property enables cfDNA to portray a "real-time" snapshot of the patient's disease state[49,69].

Analysis of ctDNA through liquid biopsy may be relevant in many clinical stages of CDK4/6 inhibitor treatment, such as prognostication, personalizing therapeutics (e.g., determining which patients should receive anti-CDK4/6 therapy or adding other agents), monitoring treatment response, and identifying resistance [Figure 1]. ctDNA can be analyzed by polymerase chain reaction (PCR)[70,71], which targets a single gene locus, or next-generation sequencing (NGS), which simultaneously profiles dozens or hundreds of genes[64,72-74]. Of relevance in ER+/HER2- advanced breast cancer, the *therascreen PIK3CA RGQ PCR kit* detects *PIK3CA* mutations to help direct PI3Ka inhibitor treatment (alpelisib)[75,76]. NGS-based liquid biopsy assays often include *PIK3CA* in addition to genes of potential relevance to resistance mechanisms to endocrine therapy, such as *ESR1* and *PTEN*[77]. Thus, although not yet established in the CDK4/6 setting, ctDNA shows considerable promise as means of biomarker detection for genotype-guided precision oncology.

GENETIC-BASED LIQUID BIOPSY BIOMARKERS OF CDK4/6 INHIBITOR EFFICACY AND RESISTANCE

Currently, there are no clinically validated liquid biopsy biomarkers to distinguish patients with differential benefits to anti-CDK4/6 therapy. Here we report the main ctDNA liquid biopsy biomarkers for CDK4/6 inhibitor treatment investigated at baseline or time of progression. The main focus of the studies outlined in this section are alterations of genes related to cell cycle regulation using ctDNA and their relationship to outcomes [Table 1].

Most studied resistance mechanisms converge on Rb modulation since it is the central target of CDK4/6 to control cell cycle progression[38]. Genetic alterations of *RB1* may cause its inactivation and confer resistance to CDK4/6 inhibitors. For example, in the PALOMA-3 trial, loss of *RB1* detected via baseline ctDNA was associated with worse PFS for patients in the palbociclib plus fulvestrant treatment group[78]. These results suggest that *RB1* alterations may be prognostic and potentially predictive; however, this study could not determine the treatment interaction effect due to the small sample size. This was further supported by an analysis of ctDNA from patients in the MONALEESA 2, 3, and 7 trials, which found that for patients with *RB1* mutations, ribociclib plus endocrine therapy did not significantly improve median PFS[79]. Furthermore, a small clinical report of three patients was the first to identify loss-of-function *RB1* mutations in ctDNA sampled at the time of acquired CDK4/6 inhibitor resistance[44]. This was later supported by an analysis of PALOMA-3 matched baseline and end-of-treatment ctDNA samples, which found loss-of-function *RB1* mutations were exclusively acquired in 4.7% of patients treated with palbociclib plus fulvestrant, suggesting they were selected for in treatment resistance. However, due to the small number of acquired *RB1* mutations, no associations were made to PFS[39]. Altogether, these results and biological support of *RB1* loss-of-function as a resistance mechanism suggest that genomic alterations of *RB1* detected in ctDNA may be predictive of CDK4/6 inhibitor resistance. However, the low prevalence of these alterations suggests other resistance mechanisms are involved, and *RB1* mutations may serve as a predictive biomarker of resistance for a limited portion of patients on CDK4/6 inhibitors.

Table 1. Summary of the main genomic alterations interrogated in ctDNA as biomarkers for CDK4/6 inhibitor resistance

Genomic alteration	ctDNA sample	Cohort	Prevalence	Technique	Main findings	Reference
RB1	Baseline	PALOMA-3: Palbociclib plus fulvestrant	27 of 156 patients (17.3%)	Target panel NGS (17 genes)	Patients in the palbociclib treatment arm with loss of *RB1* had worse median PFS compared to wild-type (exact PFS not reported)	O'Leary *et al.*[78]
	Baseline	MONALEESA 2,3,7: Ribociclib plus endocrine therapy	26 of 1,534 patients (1.7%)	Target panel NGS (~600 genes)	Patients with *RB1* mutated tumors did not have significantly different median PFS with ribociclib treatment compared to placebo (mutant: 9.2 *vs.* 3.7 months placebo *vs.* ribociclib arm)	Bertucci *et al.*[79]
	Progression	Three case reports: Palbociclib plus fulvestrant or ribociclib plus letrozole	NA	Custom library for RB1 and TP53 coding sequence/ Guardant 360 assay (73 genes)	Patients had five different loss-of-function genetic alterations of *RB1* after exposure to CDK4/6 inhibitors and coinciding with resistance	Condorelli *et al.*[44]
	Baseline and End-of-treatment	PALOMA-3: Palbociclib plus fulvestrant	Acquired in 6 of 127 patients (4.7%)	Exome sequencing/ Target panel NGS/ ddPCR	Patients exclusively acquired *RB1* alterations in the palbociclib treatment arm	O'Leary *et al.*[39]
ESR1	Baseline	PALOMA-3: Palbociclib plus fulvestrant	91 of 360 patients (25.3%)	ddPCR	Patients in the palbociclib treatment arm had similarly improved median PFS regardless of *ESR1* status, although patients in the placebo arm with *ESR1* mutations had worse PFS compared to wild-type (palbociclib arm: 9.4 *vs.* 9.5 months mutant *vs.* wild-type; placebo arm: 3.6 *vs.* 5.4 months mutant *vs.* wild-type)	Fribbens *et al.*[80]
	Baseline and End-of-treatment	PALOMA-3: Palbociclib plus fulvestrant	Acquired in 25 of 195 patients (12.8%)	Exome sequencing/ Target panel NGS/ ddPCR	Patients acquired *ESR1 Y537S* mutations in both treatment arms and were associated with improved median PFS compared to patients who did not acquire the mutation (13.7 *vs.* 7.4 months acquired *vs.* not acquired)	O'Leary *et al.*[39]
	Baseline	PALOMA-3: Palbociclib plus fulvestrant	72 of 331 patients (21.8%)	Target panel NGS (17 genes)	Patients in the placebo arm with *ESR1* mutations had worse PFS compared to wild-type (exact PFS not reported)	O'Leary *et al.*[78]
	Baseline	MONARCH-2: abemaciclib and fulvestrant	147 of 248 patients (59.3%)	ddPCR	Patients in the abemaciclib treatment arm had improved PFS regardless of *ESR1* status but observed a higher numerical median PFS in patients with *ESR1* mutant tumors compared to wild-type (abemaciclib arm: 20.7 *vs.* 15.3 months mutant *vs.* wild-type; placebo arm: 13.1 *vs.* 11.3 months mutant *vs.* wild-type). Patients with *ESR1* mutations also had improved OS (abemaciclib arm: not reached *vs.* 52.2 months mutants *vs.* wild-type; placebo arm: 42.2 *vs.* 29.4 months mutant *vs.* wild-type)	Tolaney *et al.*[81]
PIK3CA	Baseline	PALOMA-3: Palbociclib plus fulvestrant	129 of 395 patients (33%)	BEAMing assay	Patients in the palbociclib treatment arm had similarly improved PFS regardless of *PIK3CA* status (palbociclib arm: 9.5 *vs.* 9.9 months mutated *vs.* wild-type; placebo arm: 3.6 *vs.* 4.6 months mutated *vs.* wild-type)	Critstofanilli *et al.*[13]
	Baseline	MONARCH-2: abemaciclib and fulvestrant	96 of 219 patients (43.8%)	ddPCR	Patients in the abemaciclib treatment arm had similarly improved PFS regardless of *PIK3CA* status, although patients in the placebo treatment arm with *PIK3CA* mutations had worse median PFS compared to wild-type (abemaciclib arm: 17.1 *vs.* 16.9 months mutant *vs.* wild-type; placebo arm: 5.7 *vs.* 12.3 months mutant *vs.* wild-type)	Tolaney *et al.*[81]
	Baseline	Palbociclib or ribociclib plus fulvestrant or letrozole	12 of 30 patients (40%)	ddPCR	Patients treated with palbociclib or ribociclib plus endocrine therapy with *PIK3CA* mutations had a worse median PFS compared to wild-type (7.44 *vs.* 12.9 months mutant *vs.* wild-type)	Del Re *et al.*[82]
	Baseline	PALOMA-3: Palbociclib plus fulvestrant	55 of 331 patients (16.6%)	Target panel NGS (17 genes)	PIK3CA mutations were not identified as predictive (exact PFS not reported)	O'Leary *et al.*[78]
	Baseline and	PALOMA-3: Palbociclib	Acquired in 15 of	Exome sequencing/Target	Patients acquired *PIK3CA* mutations in both treatment arms and were associated with	O'Leary

	End-of-treatment	plus fulvestrant	195 patients (7.6%)	panel NGS/ddPCR	improved median PFS compared to patients who did not acquire a PIK3CA mutation (12.7 *vs.* 9.2 months acquired *vs.* not acquired)	*et al.*[39]
	Baseline	MONALEESA-7: Ribociclib plus endocrine therapy	139 of 489 patients (28%)	Target panel NGS (~600 genes)	Patients in the ribociclib treatment arm had improved median PFS compared to the placebo arm, and this was more prominent in patients with wild-type *PIK3CA* compared to *PIK3CA* mutations (ribociclib arm: 14.8 *vs.* 24.7 months mutant *vs.* wild-type; placebo arm 12.9 *vs.* 12.2 months mutant *vs.* wild-type)	Bardia *et al.*[83]
FGFR	Progression	MONALEESA-2: Ribociclib plus letrozole	20 of 427 patients (5%)	Guardant360 assay (73 genes)	Patients in the ribociclib treatment arm with *FGFR1* alterations had a worse median PFS (10.61 *vs.* 24.84 months mutant *vs.* wild-type), although significance was not achieved due to the small sample size	Formisano *et al.*[41]
	Baseline	PALOMA-3: Palbociclib plus fulvestrant	20 of 401 patients (4.9%)	Target panel NGS (17 genes)	Patients with *FGFR1* amplifications had a worse median PFS in both treatment arms (palbociclib arm: 3.9 *vs.* 12 months mutant *vs.* wild-type; placebo arm: 1.8 *vs.* 4.8 months mutant *vs.* wild-type)	O'Leary *et al.*[78]
	Baseline and End-of-treatment	PALOMA-3: Palbociclib plus fulvestrant	Acquired in 2 of 195 patients (1%)	Exome sequencing/Target panel NGS/ddPCR	Patients acquired *FGFR2* alterations with no apparent difference between treatment arms.	O'Leary *et al.*[39]
TP53	Baseline	PALOMA-3: Palbociclib plus fulvestrant	52 of 331patients (15.7%)	Target panel NGS (17 genes)	Patients with *TP53* mutations had a worse median PFS in both treatment arms, and no interaction with treatment was observed (palbociclib arm: 3.7 *vs.* 12.7 months mutant *vs.* wild-type; placebo arm: 1.8 *vs.* 5.4 months mutant *vs.* wild-type)	O'Leary *et al.*[78]
	Baseline	MONALEESA-7: Ribociclib plus endocrine therapy	92 of 489 patients (19%)	Target panel NGS (~600 genes)	Patients with *TP53* mutations had a worse median PFS in both treatment groups (ribociclib arm: 9.2 *vs.* 24.7 months mutant *vs.* wild-type; placebo arm: 7.2 *vs.* 13.0 months mutant *vs.* wild-type)	Bardia *et al.*[83]
KRAS	Baseline and on treatment	Palbociclib plus fulvestrant	66 of 106 patients (62.2%)	ddPCR	Patients treated with palbociclib and fulvestrant with baseline *KRAS* mutations had a worse median PFS compared to wild-type (3 *vs.* 17.8 months mutant *vs.* wild-type)	Raimondi *et al.*[45]
CCND1	Baseline	MONALEESA-7: Ribociclib plus endocrine therapy	51 of 489 patients (10%)	Target panel NGS (~600 genes)	Patients with *CCND1* alterations had a worse median PFS in both treatment arms and patients with altered *CCND1* also had a significant treatment interaction effect (ribociclib arm: 12.9 *vs.* 22.1 months mutant *vs.* wild-type; placebo arm: 5.5 *vs.* 11.3 months mutant *vs.* wild-type)	Bardia *et al.*[83]
MYC	Baseline	MONALEESA-7: Ribociclib plus endocrine therapy	35 of 489 patients (7.1%)	Target panel NGS (~600 genes)	Patients with *MYC* alterations had a worse median PFS in both treatment arms (ribociclib arm: 7.3 *vs.* 24.7 months mutant *vs.* wild-type; placebo arm: 7.2 *vs.* 12.9 months mutant *vs.* wild-type)	Bardia *et al.*[83]

ctDNA: Circulating tumor DNA; NGS: next-generation sequencing; PFS: progression-free survival; ddPCR: droplet digital polymerase chain reaction; OS: overall survival.

In addition to *RB1* mutations, genomic alterations in *ESR1* have been investigated due to the ER mitogenic pathway being critical to cyclin D-CDK4/6 dependence and the inactivation having a potential role in resistance to CDK4/6 inhibitors[46]. First, multiple analyses of the PALOMA-3 trial revealed that patients with baseline ctDNA *ESR1* mutations had a worse median PFS solely in the placebo treatment arm[78,80]. These findings support the role of *ESR1* mutations as a biomarker of endocrine resistance, and no treatment interaction effect was observed, indicating the lack of predictive potential of *ESR1* mutations for CDK4/6 inhibitor resistance. A later study of PALOMA-3 found that 13 of 195 patients lost *ESR1* mutations between baseline and end-of-treatment, whereas 25 patients in both treatment groups acquired the alteration, suggesting *ESR1* mutations promote resistance to fulvestrant and do not predict CDK4/6 inhibitor treatment benefit[39]. Lastly, a recent analysis of ctDNA from the MONARCH-2 trial found that abemaciclib plus fulvestrant

improved PFS regardless of *ESR1* status, but patients with *ESR1* mutations had a higher numerical median PFS in both treatment arms[81]. This study also observed an unexpectedly high prevalence of *ESR1* mutations and increased OS for patients with *ESR1* mutations. The contrast of these findings is unclear but may be explained by differences in sample size, patient criteria, and analytical techniques. Altogether, *ESR1* mutations are not a promising candidate biomarker for CDK4/6 inhibitor resistance and may be more informative for endocrine resistance.

PIK3CA mutations have also been interrogated by ctDNA due to their upstream role in cell cycle regulation through the PI3K/AKT/mTOR pathway, which interacts with estrogen receptors and potentially impacts CDK4/6 inhibitor resistance[38]. For instance, analyses of the PALOMA-3 trial found that palbociclib plus fulvestrant treatment similarly improved PFS in patients with mutated or wild-type *PIK3CA* in baseline ctDNA, indicating that *PIK3CA* genomic alterations are not predictive of CDK4/6 inhibitor benefit[13,78]. In support of this finding, a study of baseline ctDNA from the MONARCH-2 trial found patients in the abemaciclib treatment arm had similarly improved PFS regardless of *PIK3CA* mutation status[81]. In addition, patients in the placebo arm with mutant *PIK3CA* had a worse median PFS, suggesting the role of *PIK3CA* mutations in endocrine therapy resistance instead of as a biomarker of CDK4/6 inhibitor treatment. In contrast, another study of baseline ctDNA of advanced breast cancer patients treated with palbociclib or ribociclib found that patients with *PIK3CA* mutations had a shorter median PFS than wild-type[82]. Due to a lack of a control treatment arm, this study could not assess the treatment effect with *PIK3CA*. Still, these findings suggest *PIK3CA* mutations may act as a prognostic biomarker for advanced breast cancer patients receiving treatment with CDK4/6 inhibitors.

Furthermore, the lack of predictive potential of *PIK3CA* has been supported in multiple studies. An additional PALOMA-3 analysis of matched baseline and end-of-treatment ctDNA samples found that 7.6% of patients acquired *PIK3CA* mutations across both treatment arms, suggesting that *PIK3CA* mutations emerge due to fulvestrant resistance[39]. Interestingly, patients with acquired *PIK3CA* mutations had an improved PFS than those who did not; however, this trend was seen with the acquisition of any new mutation (including *ESR1*), suggesting that novel alterations are more likely to emerge in patients with a longer duration of treatment. Moreover, a recent study analyzing ctDNA from the MONALEESA-7 trial found that patients in the ribociclib treatment arm had an improved median PFS, but this was more pronounced in patients with wild-type *PIK3CA*[83]. However, this difference was not statistically significant, reinforcing the limited potential of *PIK3CA* as a predictive biomarker of CDK4/6 inhibitor resistance.

In addition, FGFR genetic alterations have been investigated in plasma ctDNA of patients treated with CDK4/6 inhibitors due to evidence of abnormal FGFR signaling, driving *CCND1* overexpression and MAPK activation, contributing to resistance[38]. One analysis of baseline ctDNA from the MONALEESA-2 trial found that for patients in the ribociclib treatment arm, *FGFR1* alterations were related to worse PFS, although significance was not achieved due to the small sample size[41]. In further support, a study of ctDNA from the PALOMA-3 trial found that *FGFR1* amplifications were associated with worse PFS in both treatment groups, suggesting that the alteration may be related to endocrine resistance[78]. Furthermore, in an assessment of pre- and post-treatment ctDNA samples from PALOMA-3, *FGFR2* was acquired in 2 of 195 patients, with no apparent difference between treatment groups[39]. Altogether these findings support FGFR alterations, specifically *FGFR1* amplifications, as a possible prognostic biomarker for patients treated with CDK4/6 inhibitors and endocrine therapy.

TP53 is a tumor suppressor gene whose product induces antiproliferative factors, such as *CDKN1A*, and its modification in ctDNA has also been investigated in many studies. For example, genomic alterations of

TP53 in ctDNA from the PALOMA-3 trial were assessed, revealing that patients with *TP53* mutations had significantly worse PFS in both treatment arms, and no interaction with treatment was observed[78]. This work also found that *TP53* mutations were connected to a distinct aggressive phenotype with more metastases but could not rule out the presence of these mutations due to clonal hematopoiesis. Moreover, a MONALEESA-7 biomarker analysis found that *TP53* mutations in baseline ctDNA were associated with progression independently from treatment with ribociclib and did not predict response to therapy[83]. Overall, these studies support *TP53* alterations as prognostic biomarkers and suggest they are not candidate predictive biomarkers of anti-CDK4/6 therapy.

In addition, genomic alterations of *KRAS* in ctDNA have recently been interrogated due to its role upstream of CDK4/6, transducing mitogenic signaling and affecting cyclin D1[38]. One study investigated *KRAS* mutations in ctDNA of 106 HR+/HER2- metastatic breast cancer patients treated with palbociclib plus fulvestrant and found that after 18 months, all patients with *KRAS* alterations had progressive disease[45]. In contrast, only one *KRAS* wild-type patient had progressed. Accordingly, patients with mutated *KRAS* had a worse median PFS compared to wild-type, supporting the potential of *KRAS* mutations as a prognostic biomarker. Further study with a control treatment group is required to determine the treatment interaction effect before conclusions can be made. For instance, previous studies on ctDNA from the PALOMA-3 trial have investigated *KRAS* mutations, but likely due to the low frequency of aberrations, have not made associations with PFS[39,78].

Many other genomic alterations have been investigated in plasma ctDNA of patients treated with CDK4/6 inhibitors due to their role around the cyclin D-CDK4/6-Rb axis or in breast cancer in general, namely *CCND1*, *CDK4*, *CDK6*, *CDKN1*, *CDKN2*, *NF1*, *ERBB2*, *AKT1*, *NRAS*, *HRAS*, *GATA3*, and *MYC*[39,78,83]. In particular, a recent study of the MONALEESA-7 samples found that patients with *CCND1* alterations in baseline ctDNA had a worse median PFS for both treatment arms, indicating the role of *CCND1* as a prognostic biomarker[83]. The benefit of ribociclib was also greater in patients with *CCND1* altered ctDNA, supporting *CCND1* as a candidate predictive biomarker. Alterations in *MYC* have also been reported as a potential prognostic biomarker for patients treated with CDK4/6 inhibitors in combination with or solo endocrine therapy[83]. Otherwise, associations with PFS and treatment have not been reported for many of the above alterations.

In summary, current work suggests *ESR1* and *PIK3CA* mutations in ctDNA have limited CDK4/6 inhibitor biomarker potential, which may be clouded due to implications with endocrine resistance. Alterations in *FGFR1*, *TP53*, and *MYC* may also be prognostic biomarkers of resistance to anti-CDK4/6 therapy, whereas *RB1*, *KRAS,* and *CCND1* may be prognostic and putative predictive biomarkers of CDK4/6 inhibitor resistance. Although numerous potential biomarkers have been identified, many studies have not confirmed the effect of interaction with treatment, and further validation is needed. While considering this, the evidence so far indicates that no singular genetic alteration will serve as an ideal prognostic or significantly predictive biomarker for CDK4/6 inhibitor efficacy or resistance. While some potential predictive biomarkers, such as *RB1* alterations, seem promising, their low prevalence indicates multifactorial resistance mechanisms. As such, analyses that only evaluate one genomic alteration are limited in assessing whether other alterations add predictive value. To assess the vast landscape of CDK4/6 inhibitor resistance mechanisms, future studies should consider a broader range of genomic alterations in ctDNA or, as discussed in later sections, expand to longitudinal monitoring to assess ctDNA dynamics or investigate epigenetic-based features.

DYNAMIC CTDNA BIOMARKERS OF CDK4/6 INHIBITOR EFFICACY AND RESISTANCE

Monitoring changes in ctDNA levels throughout treatment may reveal more prognostic and predictive information than a single time point measurement. Serial monitoring of ctDNA levels could provide a dynamic biomarker that allows personalized modifications to treatment, such as adding or switching to a more effective therapy for non-responsive patients[84]. In investigations of the utility of dynamic liquid biopsy biomarkers, questions remain concerning sampling time points, assays used, and change thresholds. This section discusses research on dynamic ctDNA biomarkers relevant to CDK4/6 inhibitor treatment [Table 2].

Dynamic ctDNA levels in response to CDK4/6 inhibitor treatment were first investigated by monitoring *PIK3CA* and *ESR1* mutant ctDNA from the PALOMA-3 trial collected at baseline, cycle one day 15, and progression[85]. *PIK3CA* and *ESR1* mutations were detected in 100 and 114 of 455 baseline samples (22% and 25.6%), with 73 and 65 matched day 15 samples, respectively. For both *PIK3CA* and *ESR1*, a significant decline in ctDNA occurred on day 15, with a more apparent decrease in mutant *PIK3CA* ctDNA in the palbociclib arm and mutant *ESR1* ctDNA in the placebo arm. This group also defined a circulating DNA ratio (CDR15) as the concentration of ctDNA on day 15 compared to baseline. They found that all patients on palbociclib had a CDR15 less than one, possibly due to the cytostatic effect of CDK4/6 inhibitors. For *PIK3CA*, patients with CDR15 above the median had a worse PFS than those below the median; however, this was not seen for *ESR1*. Using an optimal threshold determined by Harrell's c-index and Benjamini-Hochberg p-value corrections, they found that patients with a high *PIK3CA* CDR15 had an inferior median PFS than those with a low CDR15. Ultimately, this study determined that relative change in ctDNA based on commonly truncal *PIK3CA* mutations was predictive of PFS for patients treated with palbociclib and fulvestrant. Alternatively, ctDNA dynamics based on *ESR1* mutations, which are generally subclonal due to selection of prior endocrine therapy, were not predictive of clinical outcome. Altogether, these results indicate that early evaluation of ctDNA dynamics with truncal mutations may be a predictive biomarker of PFS for patients on CDK4/6 inhibitor treatment.

In addition, a subsequent study also assessed *ESR1* mutations longitudinally in the ACLINA cohort of 59 ER+/HER2- metastatic breast cancer patients treated with palbociclib plus fulvestrant[86]. They found *ESR1* mutations in 28.8% of the baseline samples, but these were not associated with PFS. In addition, they found that all patients experienced a decrease in ctDNA on day 15 relative to baseline. In contrast, on day 30, patients with early progression had increased ctDNA, and patients with longer PFS had lower or consistent ctDNA levels. They found that the presence of *ESR1* mutant ctDNA on day 30, as opposed to ctDNA clearance, was correlated with worse PFS, suggesting the potential of ctDNA detection on day 30 of treatment as a prognostic biomarker.

Further support for ctDNA ratios on day 30 as a biomarker comes from additional analysis of ctDNA from patients in the ALCINA cohort[87]. First, this study used archived tumor tissue to identify trackable mutations based on a panel of 15 driver genes. Next, they assessed serial plasma samples for 25 patients with either *PIK3CA*, *TP53*, or *AKT1* mutations and found that baseline ctDNA levels had no association with PFS. In addition, they found that all patients had a decrease in ctDNA on day 15, which was not associated with PFS. In contrast, three kinetic patterns appeared on day 30, with nine patients displaying a continuous decreased or undetected ctDNA, one patient with consistent ctDNA levels, and five patients with an increase in ctDNA. Patients with undetectable ctDNA on day 30 had an improved PFS compared to those with detectable ctDNA. Furthermore, the radiological response had high concordance with ctDNA detection, with general decreases or undetectable ctDNA for non-progressive disease compared to rising ctDNA between days 15 and 30 for progressive disease. However, this study assessed concentrations of

Table 2. Summary of dynamic ctDNA biomarkers investigated in the CDK4/6 inhibitor treatment setting

Technique	ctDNA sample time points	Genetic alteration	Cohort	Metric	Main findings	Reference
ddPCR	Baseline, cycle 1 day 15, and progression	*ESR1* and *PIK3CA*	PALOMA-3: Palbociclib plus fulvestrant	High and low CDR15 based on a threshold determined by Harrell's c-index and Benjamini-Hochberg p-value corrections	All patients in the palbociclib treatment arm had a CDR15 less than one. For *PIK3CA*, patients with a high CDR15 had a worse median PFS than those with a low CDR15 (4.1 *vs.* 11.2 months high *vs.* low)	O'Leary *et al.*[85]
ddPCR	Baseline, day 15, day 30, and progression	*ESR1*	ALCINA: Palbociclib plus fulvestrant	Ratio relative to baseline (mutant copies/mL)	All patients experienced a decrease in ctDNA on day 15 relative to baseline. Patients with early progression had increased ctDNA on day 30, and patients with longer PFS had lower or consistent ctDNA levels relative to baseline. *ESR1* mutations ctDNA on day 30, as opposed to ctDNA clearance, was correlated with worse PFS	Jeannot *et al.*[86]
ddPCR monitoring one tumor-specific mutation per patient	Baseline, day 15, day 30 and progression	*PIK3CA* (n = 21), *TP53* (n = 2), or *AKT1* (n = 2)	ALCINA: Palbociclib plus fulvestrant	Ratio relative to baseline (mutant copies/mL)	All patients had a decrease in ctDNA on day 15, but this was not associated with PFS. Patients with undetectable ctDNA on day 30 had an improved PFS compared to those with detectable ctDNA (25 *vs.* 3 months undetectable *vs.* detectable, respectively). ctDNA ratios (day 30/baseline) greater than or less than one were significantly associated with PFS	Darrigues *et al.*[87]
Guardant360 assay	Baseline, four weeks	73 genes	Palbociclib or ribociclib plus endocrine therapy	mVAFR for the 79 mutations found between baseline and week 4 assess in groups of high, medium, and low mVAFR groups and as a continuous variable	mVAFR was significantly associated with PFS, whereas single timepoint mean VAFs or absolute changes in mean VAF were not. Patients with high mVAFR had a worse median PFS than those with low mVAFR (4.2 months *vs.* not reached high *vs.* low)	Martinez-Saez *et al.*[88]
mFAST-seq	Various	Aneuploidy	CDK4/6 inhibitor plus endocrine therapy	z-score trajectories	Raised z-score trajectories were significantly related to worse PFS, whereas baseline z-scores were not predictive of progression. A single z-score increased in a consecutive blood sample at any follow-up point was not associated with PFS	Dandachi *et al.*[89]

ctDNA: Circulating tumor DNA; ddPCR: droplet digital polymerase chain reaction; CDR15: circulating DNA ratio at cycle one day 15 compared to baseline; PFS: progression-free survival; mVAFR: mean variant allele fraction ratio; mFAST-seq: modified Fast Aneuploidy Screening Test-Sequencing System.

ctDNA with respect to disease progression and found overlap in progressive versus non-progressive disease at all time points, highlighting the challenge of absolute abundance as a biomarker. Therefore, they assessed ctDNA ratios relative to baseline and found low ratios on day 15, with no significant difference in decline between patients with and without disease progression. Instead, they found that ctDNA ratios on day 30 relative to baseline distinguished patients with non-progressive disease (ratio < 1) and progressive disease (ratio > 1) and that high ctDNA ratios were associated with worse PFS. This study supports that monitoring of ctDNA is related to radiological progression and demonstrates the potential of ctDNA dynamics on day 30 relative to baseline as a prognostic biomarker for patients treated with CDK4/6 inhibitors.

In contrast to the studies highlighted above, ctDNA dynamics can be monitored using the combined signal from profiling changes in many mutated genes instead of specific mutations. For instance, a recent study assessed ctDNA levels at baseline and four weeks for 45 patients treated with CDK4/6 inhibitors and

endocrine therapy using the 73-gene Guardant360 assay[88]. This work defined a mean variant allele fraction ratio (mVAFR) as an average of mutations found between baseline and week 4 for each patient. They found that mVAFR was significantly associated with PFS, whereas single timepoint mean VAFs or absolute changes in mean VAF were not. One consideration of this study is that they assessed PFS with respect to three mVAFR groups (high, medium, and low) with cutoffs based on their cohort before assessing mVAFR as a continuous variable. Another limitation of this study was that they could not distinguish mutations from clonal hematopoiesis. Altogether, this study illustrates that early ctDNA dynamics in multiple genes may act as a biomarker to identify patients who are likely to progress on CDK4/6 inhibitors and endocrine therapy, which may provide the opportunity to modify or add treatments early on.

An additional study evaluated ctDNA using an untargeted sequencing technique, modified Fast Aneuploidy Screening Test-Sequencing System (mFAST-seq) in longitudinal samples from 49 HR+/HER2- metastatic breast cancer patients treated with CDK4/6 inhibitors[89]. In this work, associations between z-score measurements, which are surrogate measurements to ctDNA fraction, were made to clinical outcomes using joint models, which link data over a protracted period of time and time-to-event. They found that raised z-score trajectories were significantly related to worse PFS, whereas baseline z-scores were not predictive of progression. Interestingly, they found that a single rise z-score in a consecutive blood sample at any follow-up point was not associated with PFS. This study highlights the use of different assays in dynamic monitoring and reinforces that trajectories, as opposed to single time points, may be useful biomarkers of progression on anti-CDK4/6 therapy. A limitation of this approach is that mutations were not assessed, which may be relevant in the future for potential interventions or modifications to treatments. Also, this study evaluated z-score measurements at 181 time points for 49 patients, but these were not standardized and did not address the optimal time to sample. They also observed that some patients with progressive disease did not have raised z-scores over time and may be due to long intervals in sampling failing to detect an increase. This may be elucidated by shorter and more consistent sampling in future studies.

Overall, the current literature on monitoring ctDNA dynamics for patients treated with CDK4/6 inhibitors is limited. Differences in methods, patient populations, sampling time points, assays, and change thresholds lead to discordance across studies. Determining ideal change thresholds and timepoints will be essential for downstream clinical decisions. Future research should evaluate a more comprehensive range of consistent time points. Another important consideration is the way mutations are interrogated in ctDNA. Methods can vary dramatically by the number of tumor-specific markers assessed. ddPCR and related single-locus approaches can have high analytical sensitivity and specificity for a specific mutation, but other mutations and subclones that may be relevant to treatment resistance are ignored. Broader targeted panel sequencing approaches can be more robust by simultaneously interrogating multiple mutations and accounting for potential mechanisms of resistance[64,72]. Bespoke assays designed for each patient based on tumor tissue sequencing results [e.g., TARgeted DIgital Sequencing (TARDIS) and Signatera] show promise for sensitive and specific ctDNA detection[63,73,90,91], although emergent subclones that are not present in the tissue specimen can be missed[54]. Future studies may benefit from combining these approaches to achieve high analytical performance while enabling broad discovery.

EPIGENETIC-BASED LIQUID BIOPSY BIOMARKERS OF CDK4/6 INHIBITOR EFFICACY AND RESISTANCE

As outlined in the previous sections, most CDK4/6 inhibitor ctDNA studies have focused on genetic alterations (e.g., small nucleotide variants, copy number aberrations, *etc.*). In contrast, epigenetic alterations have been relatively understudied in this context. Emerging methodologies now make it easier to profile epigenetic aberrations in ctDNA, including DNA methylation, fragmentation, and histone modifications[92].

This new generation of liquid biopsy investigations has expanded the potential diagnostic use of cfDNA compared to genetic alterations on their own (e.g., providing information on the tissue of origin). Investigating epigenetic-based features has also expanded the number of cfDNA fragments of interest beyond solely mutated tumor-derived fragments. Supporting these new methods is a maturing research infrastructure (e.g., bioinformatics infrastructure, machine learning tools) for handling increasingly complex cancer liquid biopsy data[93]. Since genetic alterations in ctDNA have failed to identify clear predictive biomarkers of CDK4/6 inhibitor efficacy and resistance, there is interest in exploring the potential value of epigenetic-based biomarkers in this setting.

Phenotype of CDK4/6 inhibitor resistance and gene expression profiling

Support for epigenetic liquid biopsy approaches comes from previous work highlighting that phenotypic biomarkers that reflect transcriptomic programs are likely to predict response to CDK4/6 inhibitors. As summarized below, multiple gene expression analyses have demonstrated the effect of CDK4/6 inhibitors on proliferation and cell cycle genes, and patterns associated with resistance have been proposed.

An analysis of ER+/HER2- breast cancer patients in the NeoPalAna trial explored gene expression changes through serial tissue biopsies at baseline, cycle one day 1, cycle one day 15, and surgery. High expression of *CCNE1*, *CCND3*, and *CDKN2D* at cycle one day 15 was associated with resistance to neoadjuvant palbociclib plus anastrozole but not anastrozole alone[94]. These gene expression changes suggest that resistant tumors have continual E2F1 activity. Similarly, a gene expression analysis on tissue samples from baseline and surgery from patients treated with preoperative palbociclib found large-scale changes in genes related to proliferation and cell cycle after treatment, including a significant decrease in *CCNE2* expression in antiproliferative responders compared to nonresponders[95].

A substudy of the PALOMA-3 trial yielded partially confirmatory findings[96]. Gene expression of 2534 cancer-related genes from 302 patient tumors revealed that high expression of *CCNE1* - but not other genes related to cell cycle regulation (*CDK4*, *CDK6*, *CCND1*, and *RB1*) - was associated with resistance to palbociclib (median PFS palbociclib arm: 7.6 *vs.* 14.1 months high *vs.* low; placebo arm: 4.0 *vs.* 4.8 months high *vs.* low). The increased predictive power of *CCNE1* mRNA in metastatic biopsies suggests that sampling closer to treatment allows improved identification of predictive biomarkers, which may be facilitated by liquid biopsy. The authors also confirmed the potential role of *CCNE1* as a predictive biomarker in an independent validation cohort of breast cancer patients from the preoperative palbociclib study, where high *CCNE1* levels were associated with a decreased antiproliferative effect with palbociclib.

Further support for *CCNE1* mRNA as a predictive biomarker stems from a series of studies examining expression levels relative to those of *RB1*. One study of ER+/HER2- preclinical models found that joint decreased expression of *RB1* and increased expression of *CCNE1* commonly occurred at resistance[97]. The ratio of *CCNE1* to *RB1* was then confirmed to be associated with palbociclib resistance among patients in the NeoPalAna trial. Another study of patients treated with abemaciclib and anastrozole alone or combined within the neoMONARCH trial found that resistant tumors had higher expression levels of *CCNE1* and lower levels of *RB1*[98]. High tumor *CCNE1* expression was again associated with poor PFS among 391 patients treated with letrozole plus ribociclib in the MONALEESA-2 trial[41]; interestingly, this study also identified *FGFR1* expression as a putative biomarker for CDK4/6 inhibitors (PFS of 22 months *vs.* not reached for patients with high *vs.* low *FGFR1* expression, respectively).

One limitation of many studies correlating gene expression and PFS is that high and low expression thresholds are often determined above and below the median expression level in the cohort. Thresholds that

are biased to the study cohort make it challenging to assess the prognostic or predictive potential, make comparisons across studies, and translate biomarker development to the clinic. Regardless, the current evidence supports that gene expression may predict CDK4/6 inhibitor efficacy or resistance. Considering gene signature assays can become routine in the clinical management of ER+ breast cancer patients, such as MammaPrint and OncotypeDx, specific gene signatures that are predictive of therapy with CDK4/6 inhibitors may be defined and developed[27,29]. However, these assays require tissue samples, which have barriers to accessibility and are often limited by the quality and quantity of RNA after FFPE chemical degradation[99]. This becomes especially difficult to derive from archival tissue specimens collected years before metastatic relapse. Many of these obstacles may be overcome by assessing transcriptional and epigenetic profiles with liquid biopsy.

Opportunities of non-mutational signatures of cfDNA

The potential of epigenetic mechanisms and biomarkers for CDK4/6 inhibitor resistance has so far not been examined in detail, and investigating these avenues may provide novel insight. For instance, one recent study showed that treatment with CDK4/6 inhibitors in ER+ breast cancer causes extensive enhancer activation through activator protein-1 (AP-1) transcriptional changes. They found that the widespread chromatin remodeling with CDK4/6 inhibitor treatment may explain the effects of these drugs beyond cell cycle arrest and may be involved in early adaptations leading to resistance[100]. These epigenomic changes may also be inferred from various features of ctDNA, such as methylation, fragmentation patterns, and histone modifications [Figure 1].

DNA methylation, namely 5-methylcytosines at CpG sites, is a vital part of cell-type-specific transcriptional regulation, and methylation profiles differ between tumor and normal tissues[101]. These differential methylation patterns are maintained in plasma cfDNA and can classify cancer types with high sensitivity in both early and late-stage disease[102,103]. Differential plasma cfDNA methylation patterns can also be leveraged to delineate the contribution of various tissues to the cfDNA pool and infer the expression of genes implicated in cancer[57,103,104]. One study investigated methylation-based biomarkers in the context of CDK4/6 inhibitors by assessing the methylation status of *ESR1* in plasma cfDNA from a cohort of 49 HR+/HER2- metastatic breast cancer patients treated mostly with endocrine therapy and CDK4/6 inhibitors. Using samples from baseline and at three months, they assessed methylation levels at two main promoters with methylation-specific ddPCR. They found that a greater than 2-fold increase in promoter B or both promoters was associated with a worse prognosis[105]. While this study has various limitations, such as a small heterogeneous cohort, inability to dissect endocrine and CDK4/6 inhibitor effects, and analysis of limited loci, it paves the way for both epigenetic and dynamic cfDNA biomarker discovery approaches.

Future studies of DNA methylation in the CDK4/6 inhibitor treatment setting should consider other technical approaches. For instance, while the analysis of limited CpG sites is practical in many clinical settings for simplicity of interpretation and lower costs, expanding to genome-wide explorations may uncover novel candidate resistance biomarkers. Furthermore, bisulfite conversion is necessary for most methylation techniques but can result in excessive loss of DNA due to degradation, which is a substantial challenge for low-input cfDNA samples[106]. Forthcoming research should leverage other methods that surpass these limitations by enriching specifically for methylated fragments of cfDNA before sequencing[104,107,108]. Moreover, hydroxymethylation is a related epigenetic modification produced by TET enzymes during cytosine demethylation and acts as a marker of active promoters[109]. Though less studied in cfDNA, similar enrichment techniques have been developed to assess regions with 5-hydroxymethyl cytosines[110,111]. These approaches may be especially useful for revealing surrogates of gene expression that could confer sensitivity or resistance to CDK4/6 inhibitors.

Phenotypic information can also be inferred from distinct fragmentation patterns between ctDNA and other sources of cfDNA. The fragmentation of cfDNA is a non-random process associated with chromatin structure, gene expression, and nuclease content. Differences in nucleosome occupancy patterns at open versus closed chromatin and across varying gene expression levels affect where nucleases can access and fragment the DNA[92]. This, in turn, is reflected in the physical characteristics of the cfDNA fragments and their distribution over the genome (i.e., fragmentation features), revealing information about cell and tissue of origin. Early work on cfDNA fragments revealed differences in fragment length, a phenomenon that multiple studies have leveraged to enrich ctDNA and improve the accuracy of cancer detection[112-117].

Beyond fragment length, many other fragmentation features have also been investigated, including relative sequence coverage[118-124], end motifs[125-129], and more[130-132]. There is substantial evidence that these features convey information about DNA protection from digestion, which multiple studies have used to create cfDNA deduced nucleosome maps[58,118,120,133]. The fragmentation profiles from this work have been correlated with gene expression profiles and permitted identification of cancer type[118,120,123].

The association of fragmentation profiles with transcriptional activity may present opportunities to infer existing candidate gene expression biomarkers of CDK4/6 inhibitor resistance (e.g., *CCNE1* expression) with cfDNA in blood plasma while simultaneously permitting assessment of clinically actionable mutations to direct subsequent therapies. While promising, there remain several practical hurdles to implementing such biomarkers in the clinic. Pre-analytical variables could influence fragmentation features, and their extraction from sequencing data requires complex bioinformatics analysis. However, if these hurdles can be overcome, fragmentation-based biomarkers could greatly extend the utility of ctDNA analysis in the CDK4/6 inhibitor biomarker setting and beyond.

Another class of epigenetic modification has recently been proposed for liquid biopsy applications: post-translational modifications of nucleosomal histones in circulation. These modifications differ in euchromatin compared to heterochromatin and may signal transcriptionally active (e.g., H3K4me3, H3K36me3) or repressed (e.g., H3K27me3, H3K9me3) regions of the genome[134]. Furthermore, histone modifications may signal chromatin remodeling and transcriptional changes between cancer and healthy cells. Initial work found a global decrease in repressive histone markers across multiple cancer types[135,136]. In a recent study, cell-free chromatin immunoprecipitation and sequencing (ChIP-seq) was conducted to identify regions associated with transcriptionally active histone modifications[137]. This approach revealed signals reflective of distinct tissues and cancer types, potentially expanding the toolbox for biomarker discovery in the context of CDK4/6 inhibitors for breast cancer patients.

Overall, epigenetic profiling of cfDNA has many potential benefits that have gone mostly unexplored in the context of breast cancer resistance to CDK4/6 inhibitors. More investigation is needed to elucidate epigenetic signatures and determine which features are the most informative from the vast list growing in the literature. A combination of approaches may increase the predictive power compared to any method alone and increase the ability to direct subsequent therapies[138-140].

CONCLUSION

Biomarkers are essential for precision oncology, and despite widespread research efforts, no clinically validated biomarkers beyond breast cancer subtype have been established to guide the use of CDK4/6 inhibitors. Liquid biopsy presents many benefits for biomarker development due to being minimally invasive and encompassing the heterogeneity of metastatic sites. Accordingly, putative predictive biomarkers of resistance to treatment with CDK4/6 inhibitors have been found in ctDNA such as *RB1*,

KRAS, and *CCND1* genomic alterations; dynamic changes in ctDNA, such as changes in truncal mutations between baseline and cycle one day 15 or 30, or ctDNA clearance on day 30. Expression of E2F target genes such as *CCNE1* is also associated with resistance and could someday be reflected through emerging epigenetic ctDNA analysis methodologies. In addition, prognostic biomarkers such as *ESR1*, *PIK3CA*, *FGFR1*, *TP53*, and *MYC* alterations have also been identified.

To advance further biomarker discovery, future studies of trials or cohorts that included a control arm (e.g., endocrine therapy alone) will be especially valuable to permit the assessment of treatment interaction effects. In addition, it would be beneficial to investigate a broader range of alterations within ctDNA, given the low likelihood that a single alteration will be an ideal biomarker to the vast mechanisms of resistance. Thresholds for expression or dynamic changes in ctDNA should also be determined independently from the patient cohort in which they are studied to increase generalizability and reproducibility. Moreover, future work on ctDNA dynamics should sample a larger range of time points. There is also enormous potential for phenotype-based biomarkers, which may reflect widespread epigenetic changes and inform changes in tumor biology associated with resistance. There is a lack of established mutation-based liquid biopsy biomarkers, and features of cfDNA related to methylation, fragmentation, and histone modifications are uncharted in the context of CDK4/6 inhibitor resistance. These ctDNA epigenomic profiles should be leveraged for new biomarker discovery. Although this review has focused on the ER+/HER2- subtype of breast cancer, it is conceivable that novel predictive biomarkers could identify other patients likely to respond. Lastly, there have been substantial investigations into novel targeted agents for treatment after CDK4/6 inhibitor resistance. For instance, preclinical and clinical data indicate that treatment with novel endocrine therapies, PI3K/MAPK pathway inhibitors, downstream mitotic kinase inhibitors, and DNA-damage related inhibitors may each have a role in the treatment of CDK4/6 inhibitor-resistant disease[46]. With further development, liquid biopsy biomarkers may help direct these subsequent therapies in the future, increasing the likelihood of successful clinical development and the potential impact on patient outcomes.

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Authors' contributions

Conceptualized the manuscript: Main SC, Cescon DW, Bratman SV
Performed the literature review and wrote the original manuscript: Main SC
Supervised and edited the manuscript: Cescon DW, Bratman SV

Availability of data and materials

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Conflicts of interest

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funding to their institution from GlaxoSmithKline, Inivata, Merck, Pfizer and Roche; is a member of a trial steering committee for AstraZeneca, Merck and GlaxoSmithKline; and holds a holds a patent (US62/675,228) for methods of treating cancers characterized by a high expression level of spindle and kinetochore associated complex subunit 3 (ska3) gene. Bratman SV is inventor on patents related to cell-free DNA mutation and methylation analysis technologies that are unrelated to this work and have been licensed to Roche Molecular Diagnostics and Adela, respectively. Bratman SV is a co-founder of, has ownership in, and serves in a leadership role at Adela.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Microvesicles: the functional mediators in sorafenib resistance

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Abstract

Overcoming drug resistance in cancer therapies remains challenging, and the tumor microenvironment plays an important part in it. Microvesicles (MVs) are functional natural carriers of cellular information, participate in intercellular communication, and dynamically regulate the tumor microenvironment. They contribute to drug resistance by transferring functional molecules between cells. Conversely, due to their specific cell or tissue targeting ability, MVs are considered as carriers for therapeutic molecules to reverse drug resistance. Thus, in this mini-review, we aim to highlight the crucial role of MVs in cell-to-cell communication and therefore their diverse impact mainly on liver cancer progression and treatment. In addition, we summarize the possible mechanisms for sorafenib resistance (one of the main hurdles in hepatocellular carcinoma treatments) and recent advances in using MVs to reverse sorafenib resistance in liver cancer therapies. Identifying the functional role of MVs in cancer therapy might provide a new aspect for developing precise novel therapeutics in the future.

Keywords: Microvesicles (MVs), sorafenib resistance, tumor microenvironment, cancer therapy

INTRODUCTION

Liver cancer (LC), including hepatocellular carcinoma (HCC), hepatoblastoma, and cholangiocarcinoma, remains one of the malignant cancers worldwide with high mortality[1]. Surgery, radiotherapy and chemotherapy are considered as the main treatments for LCs at present, but their therapeutic effects are limited in advanced stages[2]. Sorafenib, an oral drug approved by the FDA for the treatment of advanced HCC, has shown excellent therapeutic effects by inhibiting tumor growth and angiogenesis *in vitro*[3,4]. However, reported resistance limits its therapeutic effects[5-9]. Microvesicles (MVs), functional mediators in cell-to-cell communication by transferring bioactive cargoes[10], play an important role in tumor progression and metastasis[11-14]. Interestingly, recent studies reported that HCC-derived MVs promoted sorafenib resistance in recipient liver cancer cells by transporting cancerous cargo compared to normal liver cell-derived MVs[15]. Thus, the role of these tiny functional vesicles needs to be cautiously studied for the in-depth understanding and successful treatment of HCC over sorafenib resistance.

Considering the necessity of overcoming sorafenib resistance while developing a successful treatment process for the challenging HCC, in the present review, we summarize the mechanism of action of sorafenib and sorafenib resistance. In addition, recent advances of MVs in intercellular communication and the intriguing contribution of MVs in cancer treatment are discussed. Especially, the review closely considers the possibilities for utilizing MVs as a potential therapeutic tool to alleviate sorafenib resistance in future cancer treatments.

SORAFENIB DRUG AND RESISTANCE

Mechanism of action of sorafenib

The revelation of the crucial involvement of Raf1 and vascular endothelial growth factor (VEGF) mediated signaling pathways in the molecular pathogenesis of liver cancer provided an interesting theoretic basis for applying sorafenib drugs to liver cancer treatment[16,17]. As a multikinase inhibitor, sorafenib strongly inhibits the tyrosine kinase Raf. Meanwhile, it has been shown to inhibit vascular endothelial growth factor receptor and platelet-derived growth factor receptor, which in turn inhibits the activation of other downstream multikinase that are normally essential for cell growth, angiogenesis, proliferation and metastasis of HCC cells [Figure 1]. Liu *et al.*[18] recorded that sorafenib inhibited the proliferation of HCC cells and reduced angiogenesis signal transduction in HCC tumor xenograft, promoting tumor cell apoptosis as well. In addition, the same therapeutic advances have also been revealed in clinical studies[19]. Nevertheless, several studies stated that the therapeutic effects of sorafenib varied among patients, some of whom experienced severe side effects[20-23].

Mechanism of sorafenib resistance

Drug resistance limits the therapeutic effects of HCC treatments. Roughly 30% of patients were reported to respond to sorafenib well at the beginning, while the subsequently acquired resistance to sorafenib usually happens within six months[24], which is far from satisfactory. As an obvious contributor that hinders the effectiveness of cancer treatment, sorafenib resistance and the possible molecular mechanisms involved in sorafenib resistance become prominently important to be discussed here.

Due to the heterogenicity of liver cancer, some patients are resistant to sorafenib primarily, while others obtain sorafenib resistance during treatment, which further limits the application of the drug and leaves the treatment process questionable. Thus, the acquired resistance of sorafenib attracted the attention of a wide range of researchers[8,25]. To date, studies on the potential mechanism of sorafenib resistance have mainly focused on the activation of drug targets and downstream signaling, regulation of cell proliferation and apoptosis signaling[5-9]. In addition, stemness and mesenchymal states of sorafenib-resistant HCC cells

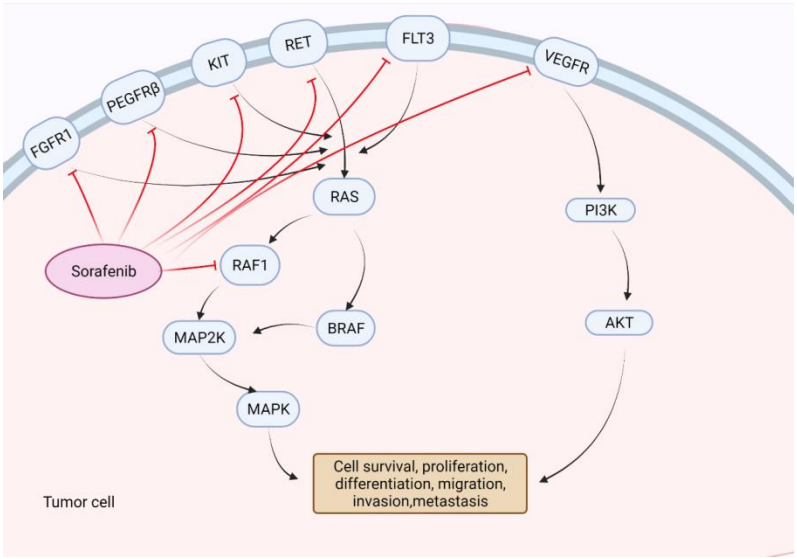

Figure 1. Mechanism of actions of sorafenib. Sorafenib inhibits tyrosine kinase receptor (VEGFR, PDGFRβ, Kit and RET) signaling and suppresses the activation of Raf, thus could suppress tumor progression by inhibiting angiogenesis and cell proliferation. Created with BioRender.com. VEGFR: Vascular endothelial growth factor receptor.

provided a new aspect of this challenging problem[26]. Altogether, the mechanism of sorafenib resistance and its influence on the treatment process remain complicated and require more research for a better understanding.

The epidermal growth factor receptor (EGFR) has been found to be overexpressed or hyperactivated in the cancer cells of most liver cancer patients as well as be the reason for continuous activation of its downstream signaling of the Ras/Raf/MEK/ERK pathway. This contributes to the abnormal proliferation of cancer cells and therefore might promote sorafenib resistance[27]. For instance, an attenuated level of phosphorylated ERK was reported to be associated with sorafenib resistance in HCC[28]. In addition, hyperactivated EGFR/HER3 and its overexpressed ligands were reported to suppress the curative effect of sorafenib by interfering with the phosphorylation of EGFR/HER3, by which the enhanced anti-proliferative and pro-apoptotic abilities of sorafenib could be achieved during the treatment[29].

A body of evidence reveals that the PI3K/Akt pathway plays an important role in sorafenib resistance[5,30]. For instance, Chen *et al.*[5] found that exposure of Huh7 liver cancer cells to a high concentration of sorafenib could result in sorafenib resistance and an accelerated expression of Akt in the treated cells. Furthermore, the PI3K/Akt pathway has been identified to have a close relationship with cell apoptosis. In the pathway, the combination of pro-survival factor and tyrosine kinase receptor activates the kinase PI3K, which triggers the downstream cascade to endorse phosphorylation of Akt and thus contributes to the suppression of cell apoptosis. In turn, inhibition of Akt could make the tumor cell more responsive to sorafenib treatment[31]. Hence, silencing of PI3K/Akt signaling with Akt inhibitor alone or with other combination therapy has gained attention for reversing sorafenib resistance for better HCC treatment[32,33]. Furthermore, Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) has been reported to be activated by sorafenib, which in turn could negatively regulate pSTAT3 and suppress transduction of JAK/STAT signaling. Dysfunctional JAK/STAT has been observed in sorafenib-resistant liver cancer cells, including induced expression of pSTAT3 and its downstream anti-apoptotic protein Mcl-1 and reduced expression of SHP-1/pSHP-1[34].

Moreover, cancer stem cells (CSCs), which represent a subpopulation of cancer cells with a self-renewal nature, are considered to participate in tumorigenesis, drug resistance, tumor metastasis and recurrence, and they are innovative targets for cancer therapy[35-37]. Label-retaining cancer cells (LRCCs) can be used to label CSCs. Xin *et al.*[38] used this methodology to observe sorafenib treated HCC cells and found that LRCCs were highly enriched in the remaining HCC cells that escaped sorafenib treatment, which strongly evidenced the resistance to sorafenib-induced cytotoxicity and apoptosis. In addition, specific ATP binding box (ABC) transporters have been reported to be highly expressed on CSCs, which control the outflow of chemical agents to protect cells from toxic compound accumulation and damage, and hence could reduce the sensitivity of cells towards drug treatment[39,40]. ABCB1 has been attested to have a close relationship with multidrug resistance; thereby, knocking out ABCB1 in drug-resistant cancer cell lines made those cells more responsive to chemotherapies[41]. It was also revealed that CSCs isolated from HCC cell lines showed resistance to sorafenib both *in vitro* and *in vivo* with abnormal IL-6/STAT3 signaling[6]. Through VEGF, liver cancer stem cells could promote tumor angiogenesis to sustain their stemness as well as drug resistance features[42]. In addition, Wnt/β-catenin signaling, one of the classic pathways involved in stemness regulation[43,44], was proven to be hyperactivated in HCC cells, resulting in β-catenin accumulation in cytoplasm and nucleus, which finally led to enhanced self-renewal ability of CSCs[45,46]. Inhibition of Wnt signaling has been shown to be beneficial to CSC clearance and tumor development[46,47].

Dual biotransformation routes including oxidation and glucuronidation were witnessed in the sorafenib metabolism[48-50]. After hepatocellular uptake, sorafenib was N-oxidized by CYP3A4, one of the drug-metabolizing enzymes, to the pharmacologically active sorafenib-N-oxide metabolites[51,52]. However, CYP3A4 was identified as poorly expressed in liver cancer[53]. Well-studied oncomiRs (e.g., miR 21, miR-142 and miR-27b) overexpressed in HCC, which could be transferred by tumor-derived microvesicles (TMVs), were proved to be negatively associated with CYP3A4 mRNA in human liver[54], which might downregulate the expression of this main enzyme and thus could inhibit the active biotransformation of sorafenib drug. Apart from oxidation, sorafenib underwent glucuronidation, mainly mediated by UGT1A1 and UGT1A9, to inactive glucuronide metabolites[48,55]. A recent study revealed that sorafenib inhibits the above-mentioned UGT enzyme[56], which might be the blockage for sorafenib secretion to bile and later systemic circulation and clearance. Taken together, the insufficient oxidation and complex interaction between sorafenib and UGT enzymes need further investigation for a deep understanding of their role in resistance.

Anti-angiogenesis is one of the therapeutic effects of sorafenib, while the tumor vessel depletion along with pericyte could induce hypoxia and allow the maintenance and enhancement of CSCs in HCC[57,58]. Apart from specific niches, different stroma cells in the microenvironment render sorafenib sensitivity[59], where MVs play a significant role.

MVS: THE TINY MEDIATORS IN INTERCELLULAR COMMUNICATION

MVs, a subpopulation of extracellular vesicles (EVs), act as functional mediators by transferring bioactive molecules among various types of cells and thus have been considered as potential candidates in intercellular communication[10]. Through unconventional secretion mechanisms, eukaryotic cells were reported to release membrane-enclosed vesicles both *in vivo* and *in vitro*[60]. In general, MVs can be collected from cell culture media and blood from animals and patients via ultracentrifuge method, filtration, or commercial kits[61]. MVs are also identified as shedding microvesicles or microparticles, budding directly from the plasma membrane and are 100 nm-1000 nm in diameter. They participate in cell-to-cell communication by carrying the information from parental cells to others and orchestrate complicated physiological and pathological processes[14,62]. At the molecular level, symmetrical perturbation of membrane lipids leads to the surface expression of phospholipid serine acid pairs, which translocate from the inner

lobule to the outer surface lobule of the membrane bilayer through special biological enzymes (floppases, flippases and scramblases), contributing to MVs formation[63]. However, the detailed mechanism of MVs formation and shedding remains to be further studied.

Evidence reveals that most MVs tend to be decomposed after shedding to release their cargo[64]. Especially, various cytoplasmic proteins, excluded from the classic signal-peptide secretion pathways, are discharged out regularly through MV decomposition[65]. For instance, fibroblast growth factor 2 (FGF2) was proved to be released in response to the breakdown of MVs derived from neurons, HCC and endothelial cells[66]. Similarly, MVs secreted by dendritic cells, macrophages and microglias have been demonstrated to release the pro-inflammatory cytokine interleukin 1B (IL-1B); IL-1B has also been found to aggregate in MVs along with proteases such as caspase 1[67]. A study on tumor-stromal interactions noted that when MVs were co-released with extracellular matrix metalloproteinase inducer (EMMPRIN/CD147), they prompted lung cancer cells to obtain increased mobility and invasion ability by promoting metalloproteinase to capture and digest extracellular matrix (ECM)[68]. In addition, Kornek *et al.*[69] demonstrated that EMMPRIN released by circulating T cell-derived MVs accelerated hepatic stellate cells to transform into a fibrolytic phenotype, which promotes ECM degradation.

Conversely, MVs are able to stimulate the receptor cells to complete signal transduction through their surface ligands. It has been confirmed that MVs derived from platelet arouse the intra-hemopoietic signal cascade by ligands such as CD40L/PF-4 and thus cause the proliferation and survival of hematopoietic cells[70]. Simultaneously, the adhesion molecules could be transferred to hematopoietic cells via MVs to enhance their adhesion to fibrinogen or endothelium[70]. Proteomic and transcriptomic studies have shown that MVs are natural vectors for transferring bioactive molecules, including protein, mRNA and miRNA, between cells[71], effectively facilitating intercellular communication[72,73]. In addition, MVs hold natural stability in the blood, low immunogenicity and special homing ability to specific organs or tissues[74]. Evidence shows that vesicle-associated integrins α6β4 and α6β1 are correlated with lung metastasis while αV β5 with liver metastasis, which indicates the various origins of MVs encoded with different tags for the corresponding cells or organ targeting[74]. Moreover, it has also been demonstrated that MVs could possibly transport even complete organelles (e.g., mitochondria) to target cells[75], which declares its potential to act as a promising novel drug carrier system.

Several studies revealed that TMVs transport cancerous molecules to recipient cells, which eventually contribute to tumor progression and metastasis[11-14,76] [Figure 2]. Interestingly, the transport of anti-cancer drugs out of ovarian cancer cells through their secreted EVs supported the role of EVs in cancer progression and treatment. Conversely, the observation of more sorafenib resistance in HCC tumors that were treated with tumor cell-derived MVs added evidence to the noticeable link between MVs and sorafenib resistance[15,78].

THE DYNAMIC IMPACT OF MVS ON SORAFENIB RESISTANCE

MV-mediated sorafenib resistance

The physiological status of parental cells decides the composition of their secreted MVs, having direct or indirect effects on the uptake of MVs by recipient cells. Tumor cells that undergo hypoxic stress and obtain stemness or mesenchymal state for survival[57,58] promote tumor progression and therapy resistance. By inducing hypoxic stress, the hypoxia-inducible factor-1 α could stimulate the release of MVs along with the modulation of its packed cargoes[79]. Similarly, Wang *et al.*[80] reported that chemotherapeutic agents could enhance the secretion of ABCB-1-enriched EVs, which promote resistant phenotype transformation in recipient cells. Specifically, growth and pro-angiogenic factors transferred between CSCs and vascular

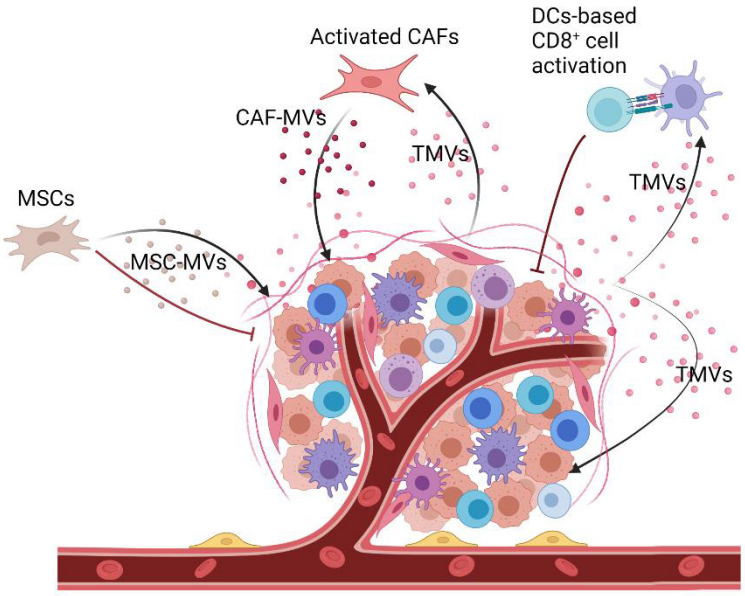

Figure 2. The bidirectional role of TMVs in tumor development. TMVs derived from tumor cells could promote tumor progression via transferring cancerous molecules[13,62]. Simultaneously, they act as a functional regulator to modulate DC cells based on $CD8^+$ T cell activation, which provokes cytotoxic T cell infiltration and inhibits tumor development[77]. Created with BioRender.com. TMVs: Tumor-derived microvesicles.

niches via vesicles under hypoxia were witnessed[81] to limit the therapeutic effect of sorafenib.

Cancer-associated fibroblasts (CAFs), one of the main stroma cells, advance the self-renewal feature of CSCs in HCC and thus could induce sorafenib resistance by secretion of hepatocyte growth factor (HGF)[82,83]. A reduction in cancer cell stemness was recorded by inhibiting the paracrine behavior of CAFs[84]. Conversely, TMVs have been shown to activate CAFs to improve the mobility of tumor cells, while the activated fibroblasts could secret MVs and in turn facilitate tumor progression[12,85]. Moreover, small GTPases and RHO-associated protein kinase, key factors in the biogenesis of MVs[73], were found to be highly expressed in both CAFs and cancer cells[86], which indicates the possible role of CAFs in the alteration of MVs secretion.

As the messengers and mediators between cells, MVs could regulate the sorafenib sensitivity in the recipient cells via their cargoes [Table 1]. For example, miRNAs involved in the regulation of multiple mRNA targets in recipient cells were found to be enriched in TMVs[94-97]. In recent years, our group also found that HCC cell-derived MVs could increase sorafenib drug resistance by inducing FOXM1 expression via miR-25 transferred to the recipient liver cancer cells from the parent cells both *in vitro* and *in vivo*[15]. Similarly, long non-coding RNA (lnc-ROR and lnc-VLDLR)-enriched HCC cells-secreted vesicles (especially after sorafenib exposure) were proved to reduce apoptosis induced by chemotherapy[88,89]. However, MVs released from modified adipose tissue-derived MSCs have been shown to carry miR-199a/miR-122 and have the ability to improve chemosensitivity in HCC[90,91]. Elevated miR-214 in human cerebral endothelial cell-released vesicles was noted to enhance the anti-tumor efficacy of sorafenib[92] [Figure 3]. However, complete genomic and proteomic analyses of preclinical MVs cargoes need to be performed in the future.

Targeting MVs to reverse sorafenib resistance

Recently, the promising outcome of targeted gene therapy has motivated more researchers to meet sorafenib resistance in HCC. A lower expression of miR-34 has been found to have a direct relationship

Table 1. Key molecules transferred by MVs modulating sorafenib resistance

Key molecules	MVs sources	Function	Ref.
miR-25	HCC cells	Increase sorafenib resistance	[15]
miR-494-3p	GOLPH3 overexpressed HCC cells	Promote the angiogenesis ability of HUVECs and induce sorafenib resistance in HCC cells	[87]
lnc-ROR	HCC cells	Reduce chemotherapy-induced cell death	[88]
lnc-VLDLR	HCC cells	Reduce chemotherapy-induced cell death	[89]
miR-199a	Adipose tissue derived MSCs	Increase chemosensitivity in HCC	[90]
miR-122	Adipose tissue derived MSCs	Increase chemosensitivity in HCC	[91]
miR-214	Human cerebral endothelial cells	Sensitize HCC cells to sorafenib treatment	[92]
siGRP78	Modified bone-marrow-derived mesenchymal stem cells	Suppress sorafenib resistance	[93]

MVs: Microvesicles; HCC: hepatocellular carcinoma.

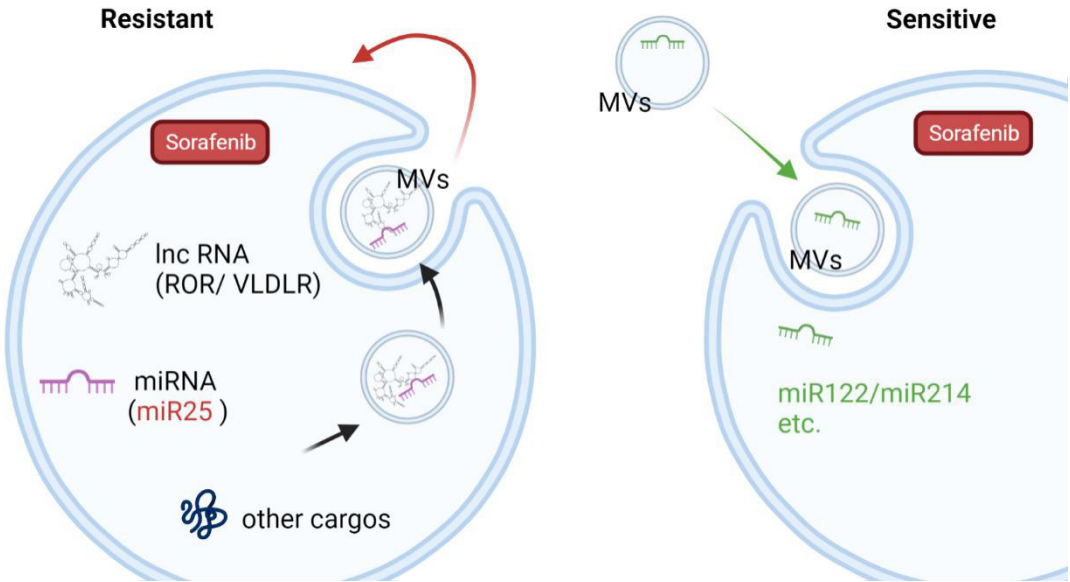

Figure 3. The dynamic role of MVs in sorafenib resistance modulation. MVs transfer biomolecules including miRNAs and lncRNAs to recipient cells and regulate their sensitivity to sorafenib. Created with BioRender.com. MVs: Microvesicles.

with patient survival, while the restoration of the miR-34a expression level in HCC cells has been noted to significantly downregulate the expression of BCL2 and enhance the cells' sensitivity towards sorafenib-induced apoptosis and toxicity[7]. Dai *et al.*[98] found that BikDD gene therapy combined with low-dose sorafenib could enhance the anti-tumor efficacy of sorafenib and improve the survival rate of tumor-bearing mice. Furthermore, the simultaneous release of sorafenib and USP22 shRNA (shUSP22) by galactose-modified lipopolysaccharide revealed that the encapsulated sorafenib along with shUSP22 could obtain a synergistic anti-proliferative effect in HCC cells by inducing reactive oxygen cascade to promote the release of shUSP22 and inhibit the expression of USP22 in HCC cells, which promoted the accumulation of sorafenib by downregulating the expression level of multidrug resistance-related proteins[99].

Several emerging studies reported utilizing modified EVs to overcome chemoresistance during cancer treatments. As a naturally secreted nanoparticle, MVs exhibited excellent specific tissue homing ability and acted as good functional carriers for various therapeutic molecules[15,74,100]. To achieve bioactive and

continuous drug containing MVs, the pre-loading strategy has been applied to modify donor cells. This strategy includes incorporating cargo into donor cells, so the donor cells could encapsulate the cargo during secretion production. Both biologically produced molecules (proteins and nucleic acids) and synthetic chemicals can be encapsulated into secreted vesicles[2]. By combined regulation of Akt/mTOR/PTEN, EVs secreted from human liver stem cells were proved to upgrade CSCs' sensitivity to sorafenib[101]. Lou *et al.*[91] stated that treating HCCs with exosomes derived from miR-122 overexpressed AMSCs rendered HCCs more responsive to sorafenib. In the same way, miR-744-enriched exosomes have been demonstrated to be a potential tool for reducing sorafenib resistance[102]. Apart from utilizing the intrinsic homing ability, controllable targeting methods, including genetic engineering (pre-targeting)[103] and conjugation of ligands (post-targeting)[104], have also been developed for further clinical research. Engineering the donor cells by inserting sequences that encode desired targeting protein makes the affibody express on the surface of MVs[105]. In this way, our group also recently recorded that CRISPR system-carried engineered MVs derived from HEK293 cells could precisely disrupt IQGAP1, which is involved in PI3K/Akt signaling, and achieved an enhanced synergistic anti-cancer effect when combined with sorafenib treatment[106].

CONCLUSION

Sorafenib resistance remains challenging in HCC treatment, while targeting the suspicious genes involved in the resistance mechanism to shut off the pathological cycle provides a new chance for advancing the cancer treatment as well as the existing anti-cancer drug[107]. For instance, by delivering sorafenib and CRISPR system via nanoparticles, Zhang *et al.*[108] achieved effective modification of EGFR, following synergistic inhibition of angiogenesis and tumor cell proliferation. Even though emerging synthetic nanomaterials have shown great abilities as therapeutic vectors, their long-term safety remains uncertain.

MVs, cell-derived natural carriers, are advanced promising gene therapy vehicles that could specifically deliver therapeutic bioactive molecules[109]. Platelet-derived MVs have been reported to hold good biocompatibility to target leukemia cells naturally and thus have been utilized as targeted delivery vehicles for multiple drugs in leukemia treatment[110]. Macrophage-derived MVs have been noted to have the ability to transport cargoes specifically to hematopoietic stem and progenitor cells (HSPCs) both *in vivo* and *in vitro*. In addition, Kao *et al.*[100] showed that plasmids and small RNAs (miRNA and siRNA) that were encapsulated by macrophage-derived MVs exhibit successful modification of heat shock protein in the recipient HSPCs. In addition, MVs were used to transport engineered minicircle DNA by researchers to achieve good gene-mediated prodrug transformation and effectively promote tumor cell death in breast cancer cells as well as mouse models[111]. However, the unexpected combination, modification, and dissociation of the above-mentioned therapeutic nucleic acids are the main concerns in gene manipulation techniques, in which the off-target effect needs to be well controlled in future clinical trials.

Considering the challenges of sorafenib resistance and MVs as the functional regulator in cancer microenvironment, in this review, we discuss the potential role of MVs in sorafenib resistance. On the one hand, in cancer progression, MVs transport bioactive molecules to participate in cell-to-cell communication, making the microenvironment favor tumor growth, which could facilitate the tumor cells to be resistant to sorafenib treatment. On the other hand, therapeutic molecules could be transferred specifically to tumor cells to alleviate the sorafenib resistance via MVs. Thus, a better understanding of these tiny players' role in tumor microenvironment and cancer progression is necessary to appropriately use this double-edged sword for precise anti-cancer therapies in the future.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: He C, Jaffar Ali D
Provided overview: Sun B, Sun BC, Xiao ZD

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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An overview of resistance to chemotherapy in osteosarcoma and future perspectives

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Abstract

Osteosarcoma (OS) is the most common type of bone sarcoma. Despite the availability of multimodal treatment with surgery and chemotherapy, the clinical results remain unsatisfactory. The main reason for the poor outcomes in patients with OS is the development of resistance to methotrexate, cisplatin, doxorubicin, and ifosfamide. Molecular and cellular mechanisms associated with resistance to chemotherapy include DNA repair and cell-cycle alterations, enhanced drug efflux, increased detoxification, resistance to apoptosis, autophagy, tumor extracellular matrix, and angiogenesis. This versatility of cells to generate chemoresistance has motivated the use of anti-angiogenic therapy based on tyrosine kinase inhibitors. This approach has shown that other therapies, along with standard chemotherapy, can improve responses to therapy in patients with OS. Moreover, microRNAs may act as predictors of drug resistance in OS. This review provides insight into the molecular and cellular mechanisms involved in the development of resistance during the treatment of OS and discusses promising novel therapies (e.g., afatinib and palbociclib) for overcoming resistance to chemotherapy in OS.

Keywords: Osteosarcoma, therapy resistance, tyrosine kinase inhibitor, tumoral extracellular microenvironment, cell cycle

INTRODUCTION

Osteosarcoma (OS) is the most common type of bone sarcoma; these tumors form a heterogeneous group of malignant neoplasms characterized by the production of osteoid matrix[1,2]. OS is disseminated by the hematologic route, and the lungs are the main site of metastasis[2-4]. It is considered that up to 70% of patients with OS have at least one micrometastatic disease at this site at the time of diagnosis[5]. The addition of chemotherapy to the treatment of patients with localized disease OS improves their prognosis, increasing their survival rate by up to 70%[1,4,6]. However, metastatic patients continue to have a poor prognosis at 20%-30%[7].

The current standard treatment modalities for OS are neoadjuvant chemotherapy, surgery and adjuvant chemotherapy. Chemotherapy is based on different combinations of doxorubicin (DOX), cisplatin, and methotrexate (MTX). The administration of the latter depends on the age of the patient and may be combined with ifosfamide (IFO), etoposide, *etc*.[8,9]. The current chemotherapy scheme was first introduced in the late 1970s and remains virtually unchanged despite numerous efforts to improve treatment outcomes[10]. One of the current clinical challenges is drug resistance, which may be inherent or acquired[11]. Additionally, patients with poor response and refractory disease (i.e., recurrent or progressive) have a bleak prognosis due to the limited number of effective options in second- or third-line chemotherapy[12-14].

This situation has generated several lines of research to identify the pathophysiological mechanisms by which OS cells develop drug resistance and, thus, devise new strategies based on the utilization of biomarkers or targeted therapies[11,15,16]. The high degree of heterogeneity observed in OS renders therapy even more problematic; for instance, the discovery of reliable biomarkers, recognition of the mechanism of recurrence, and identification of cell types that cause OS pose challenges to investigators[17]. Cancer cells can utilize various mechanisms to circumvent or counteract the cytotoxic stimuli induced by anticancer therapy: (1) impairment of drug transport and enhancement of drug efflux; (2) increase in deoxyribonucleic acid (DNA) repair; (3) alterations in cell cycle and apoptosis; (4) activation of signal transduction pathways; (5) the tumor microenvironment (TME) and angiogenesis; (6) autophagy; (7) micro-RNA; and (8) maintenance of a stem cell-like phenotype[18]. The purpose of the present narrative review is to summarize the advances achieved thus far in this setting and present some perspectives for the treatment of OS in the future using novel drug combinations.

IMPAIRED INTRACELLULAR ACCUMULATION

OS cells decrease drug accumulation to overcome the cytotoxic effects of chemotherapeutic agents. Insufficient drug transport can be related to reduced folate carriers on the cell membrane, increasing drug efflux, or inducing alterations in target enzymes[18].

Insufficient drug transport

Impaired drug transport is a well-described mechanism of resistance to chemotherapy in OS. In particular, this is achieved through a decrease in transporters present on the membrane of tumor cells. MTX is an anti-folate that uses the reduced folate carrier (RFC) to enter OS cells. Following entry, MTX is polyglutamylated to be retained in the cells. Subsequently, MTX inhibits dihydrofolate reductase (DHFR) - the enzyme that converts the dihydrofolate to tetrahydrofolate - which is a one-carbon donor for the de novo synthesis of purine and thymidine. DHFR is essential for the de novo synthesis of DNA, and the interaction between

MTX and DHFR prevents DNA synthesis[18]. Treatment-resistant OS cells have reduced expression of RFC[19]. The decreased expression of RFC in the tumor is associated with the development of resistance to MTX and poor histological responses to preoperative chemotherapy[20]. Drugs, such as trimetrexate, do not require RFC for transport. In a phase 2 clinical trial including patients with relapsed OS, toxicity was acceptable, myelosuppression was the major side effect, objective response was 8% (n = 39; complete response = 1, partial response = 2, mixed response = 1, and stable disease = 8)[21-23]. The combination of trimetrexate with high-dose MTX is currently being tested in a phase 1 trial to evaluate the efficacy, safety profile, and most appropriate dose of trimetrexate (clinical trial identifier: NCT00119301)[24] [Figure 1].

Enhancement of drug efflux

Drug resistance in OS has been linked to an increase in drug efflux, particularly for DOX. This acquired resistance mechanism is termed multidrug resistance (MDR) and is associated with the overexpression of members of the ATP-binding cassette (ABC) family of efflux transporters. Principally, this process involves the multidrug resistance protein 1 (*MDR1*) gene, which encodes P-glycoprotein (P-gp), also termed MDR1 or ATP-binding cassette subfamily B member 1 (ABCB1). These transporters are active pumps for drugs, such as DOX[18,25]. OS cells exhibit high expression levels of ABC transporters, such as MDR1, and are resistant to DOX. An association between MDR1 overexpression and reduced DOX accumulation has been reported. In patients with OS, the overexpression of ABC transporters is associated with poor response to chemotherapeutic agents (e.g., DOX) and worse clinical outcomes[20,26]. In preclinical models, the knockout of ABCB1 restored sensitivity to DOX. Drugs, such as trabectedin, may inhibit the transcriptional activation of MDR1; trabectedin modulates gene expression in a promoter manner, affecting the MDR1 gene promoter, thereby emerging as a potential alternative therapeutic strategy for the restoration of sensitivity to DOX[27].

Alterations in target enzymes

Secondary to alterations in enzymes, increased levels of target enzymes or degreased drug affinity can result in resistance to chemotherapeutic agents. MTX-resistant OS cell lines overexpress DHFR, and high expression of DHFR is also observed in OS metastasis[28,29]. DNA topoisomerases II (TOP2) are nuclear enzymes involved in the regulation of DNA topology; TOP2 forms a homodimer that functions by cleaving double-stranded DNA, coiling a second DNA duplex through the gap, and re-binding the strands. TOP2 is essential for cell replication and viability and is recognized as a target of doxorubicin[30]. In human cells, two isoforms are described, α and β; in several tumors, low expression of *TOP2B i*s described in DOX resistance, particularly OS DOX-resistant cells had reduced expression of *TOP2B* compared with DOX-sensitive cells[31]. Amplification or deletion in TOP2 genes has been described in OS patients. *TOP2B* was deleted in 40.5% of cases and is related to worse event-free survival. *TOP2A* is amplificated in 21% and deleted in 25% of OS tumors. Both gene alterations (amplification and deletion) in *TOP2A* have been correlated with a good response to neoadjuvant chemotherapy and also related to DOX resistance[32]. Exploring these enzymes in clinical trials could give us possible utility as biomarkers to predict chemotherapy response.

Several mutations in tumor suppressor genes and other pathways are involved in OS failure to chemotherapy. The most frequent gene altered in this pathology is tumor suppression gene *P53*, and mutations in other cancer drivers such as *RB1, ATRX2, DLG23, RUNX2, WRN, RECQL4, CDKN2A/B, BLM, PTEN,* and PI3K/AKT/mTOR pathway members are described in OS tumors. Some of these genes are involved in cell cycle control and DNA damage repair[33].

OS AND ALTERATIONS IN DNA REPAIR

It is established that chemotherapeutic agents cause DNA damage that leads to cell death. However, tumor

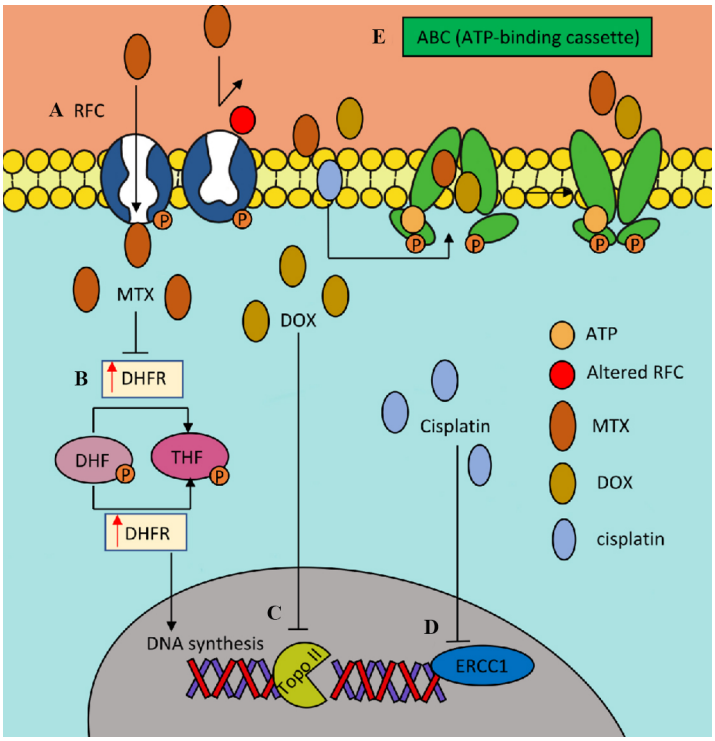

Figure 1. Reduced intracellular drug accumulation. (A) Decreased expression of the reduced folate transporter (RFC) or decreasing transporter function promotes methotrexate resistance. (B) Overexpression of dihydrofolate reductase (DHFR) responsible for the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) or alterations in the binding affinity of DHFR for methotrexate (MTX) are related with MTX resistance. (C) Decreased expression or mutation of topoisomerase II (Topo II) is associated with doxorubicin (DOX) resistance. (D) High levels of excision repair cross-complement protein 1 (ERCC1) proteins are related to cisplatin resistance. (E) Increased expression of multidrug resistance gene encoding the ATP-binding cassette (ABC) family leads to increased drug efflux and decreased intracellular drug accumulation increasing resistance to DOX and MTX.

cells can occasionally resist treatment by enhancing their DNA repair pathway. Cisplatin is one of the most studied drugs for which resistance due to an enhanced DNA repair in OS has been reported[18,34,35]. Nucleotide excision repair (NER) is an important DNA damage removal pathway. It is implicated in cancer progression and response to platinum-based chemotherapy. Additionally, NER is one of the most studied pathways in the development of drug resistance in OS. NER proteins can repair chemical drug-induced DNA damage. Members of the NER pathway that have been studied thus far include DNA excision repair proteins and excision repair cross-complement proteins (ERCC); these endonucleases are involved in the excision of the lesion, followed by DNA replication and repair process. Defects in NER accumulate DNA damage favoring cancer phenotype. Single nucleotide polymorphism (SNPs) of *ERCC1* and *ERCC2* genes may decrease the expression of *ERCC1* and *ERCC2* genes and are associated with chemotherapy response for OS, particularly reduced resistance to cisplatin-based chemotherapy[36,37]. In addition, silencing of the *ERCC1*, *ERCC2*, *ERCC3*, and *ERCC4* genes increases sensitivity in resistant OS cell cultures (U2OS/cisplatin 300 and U2OS/cisplatin 1)[35]. Base excision repair (BER) is also involved in OS. BER is primarily active in DNA damage caused by small chemical alterations or base loss due to hydrolysis of glycosyl DNA bonds. DNA damage is removed by glycosylases, a complex formed by APEX1 endonuclease, poly(ADP-ribose) polymerase (PARP), and DNA ligase, and XRCC1 recognized the bases sites. Different members of BER are involved in OS; apurinic/apyrimidinic endonuclease (APE-1) has been associated with shorter survival in patients with OS[18,37].

DNA double-strand breaks (DSBs) represent a challenge to genomic integrity. DSBs are detected by a cascade of proteins, involving the process of homologous recombination (HR) or nonhomologous end-join (NHEJ). DSBs activate pathways such as ATM and ATR that result in the phosphorylation of multiple targets, including histone H2AX and checkpoint mediator proteins (CHK) 1 and 2, to finally activate P53. ATR/CHK1 signaling is linked with the activation of BRCA2, recruiting BRCA1 and RAD51, which form filaments on the single-stranded DNA to repair the site of the DNA damage. *BRCA1* and *-2* mutations lead to impairment to repair DSBs mediated by HR. Deficiency of inhibition of PARP1 in normal cells results in an impairment of the BER response, causing lesions that should be repaired by BER activating HR pathway; however, OS presents mutations in different "BRCA" genes such *PALB2, CHEK2, PTEN,* and *ATM,* resulting in chromosomal instability analogous to *BRCA1/2* mutations[33], which make it difficult to repair DNA lesions. Exposing BRCA1/2-deficient cells to PARP inhibition results in lethal DNA damage accumulation; consequently, PARP inhibition results in the targeted tumor cell death in BRCA-deficient cancer. Preclinical data suggest the effect of PARP inhibitors in OS MNNG/HOS cells carrying disruptive gain in the *PTEN* gene and deletion of *ATM* gene; the combination of talazoparib, a phase 3 PARP inhibitor, with topoisomerase I inhibitor SN-38 considerably decreases the viability of MNNG/HOS cells, and olaparib was tested in HOS and MG-63 cells with good results. In addition to these preclinical data, it seems that the inhibition of PARP1/2 in *BRCA1/2* tumor suppressor mutated cells is involved in drug resistance in OS. The use of olaparib (a PARP1 inhibitor) sensitizes OS cell lines to treatment with DOX[38]. These types of in vitro studies led to PARP inhibitors being tested in the clinical setting. The ongoing NCT03233204 study investigates the combination of olaparib plus DOX in patients with refractory OS[39]. The TOMAS trial tested olaparib and trabectedin in sarcomas. This trial included seven patients with bone cancer; unfortunately, none of the patients showed an objective or clinical response[40], probably due to the small number of patients included in the trial. Furthermore, other combinations, such as olaparib plus ceralasertib (AZD6738), an orally available morpholino-pyrimidine-based inhibitor of ataxia telangiectasia and rad 3-related (ATR) kinase, were tested in this trial (clinical trial identifier: NCT04417062)[41]. Recently, the RB pathway has been described as PARP inhibitor (PARPi) sensitive; RB1-defective OS revealed hypersensitivity to the PARPi olaparib, and RB1-defective OS cells may yield BRCAness/HR defects by inducing RAD51 recruitment. Olaparib increases H2AX histone marker and CHK1 phosphorylation[42]. RB is highly mutated in OS patients. The MATCH trial is a precision medicine cancer treatment clinical trial where patients are assigned to receive treatment based on their genetic changes. This trial involves children, adolescents, and young adults with advanced cancers, including rare cancers such as osteosarcoma. Olaparib will be tested in patients with defects in DNA damage repair genes and BRCA1/2 mutations. We await the evolution in patients with RB1-mutated disease if they are included[39,43]. The potential benefits of anti-PARP treatment will have to wait for the publication of the results of this trial to evaluate future areas of opportunity with this inhibitor.

CELL CYCLE AND APOPTOSIS DISTURBANCES

DNA damage is one of the principal action mechanisms of chemotherapy, leading to cell death through apoptosis. For their survival, tumor cells arrest their cell cycle to repair DNA damage, thereby evading apoptosis[44]. Disturbances in the cell cycle and apoptosis are involved in the development of resistance to chemotherapy in OS cells. For example, following the overexpression of murine double minute 2 (*MDM2*) (a downstream mediator of p53) in tumor cells, p53-mediated apoptosis is inhibited, and cells develop resistance to DNA-damaging agents[18,45]. The amplification of *MDM2* is present in 20% of OS cells[46].

Cyclin-dependent kinase 4

Cyclin-dependent kinase 4 (CDK4) is an enzyme encoded by the *CDK4* gene. The activity of this kinase is restricted to the G1-S phase, and it is responsible for the phosphorylation of the retinoblastoma (RB) protein. The amplification of *CDK4* is found in 20% of OS cells[46]. In a study of 50 pediatric and adolescent

patients diagnosed with high-grade OS, the copy number analysis detected a recurrent gain of chromosome 12q14.1. This observation was more frequent in the poor responder cohort than in the good responder cohort, where the *CDK4* gene was associated with copy number gains[47]. CDK4 is highly expressed in human OS tissues and cell lines compared with normal human osteoblasts. Elevated CDK4 expression is associated with metastasis and poor prognosis in patients with OS[48]. Overexpression of CDK4 is also related to resistance to cisplatin; treatment of U2OS cells overexpressing CDK4 with CDK4/6 inhibitor palbociclib facilitates apoptosis and decreases cell viability in a dose-dependent manner[47]. The combination of sorafenib (a multikinase inhibitor) and palbociclib in a cisplatin-resistant, patient-derived, orthotopic, xenograft mouse model of OS resulted in tumor regression and enhanced tumor necrosis[49]. Patients with co-amplification of *MDM2* and *CDK4* were treated with the *MDM2* inhibitor ALRN-6924 and palbociclib; the study included ten liposarcomas, one OS, and one glioblastoma. The study concluded that the combination of ALRN-6924 and palbociclib was feasible and well tolerated[50]. Palbociclib is currently tested in pediatric tumors, including osteosarcoma, with activated alterations in cell cycle genes in the pediatric MATCH treatment trial (NCT03526250)[51]. Abemaciclib is an inhibitor of CDK4/6; Wang *et al.* evaluated the efficacy of abemaciclib in OS cells and an animal model[52]. Abemaciclib inhibited growth and anchorage-independent colony formation of OS cells and inhibited tumor formation and growth in a dose-dependent manner in the animal model. Abemaciclib combined with DOX results in much greater efficacy than DOX alone in inhibiting tumor growth; it acts by suppressing the CD4/6-Cyclin D-Rb pathway. A clinical trial with OS and abemaciclib is ongoing (NCT04040205)[52,53].

Myeloid cell leukemia-1 protein and its potential role in OS

Cell death signaling is orchestrated by members of the B cell lymphoma 2 (BCL2) family that contains antiapoptotic proteins (e.g., BCL2 and BCLXL) and proapoptotic proteins [e.g., BCL2 associated X (BAX)]. Inhibition of BCL1/BCLXL enhanced the chemosensitivity of OS to DOX and cisplatin. Myeloid cell leukemia-1 (MCL-1) is a pro-survival member of the BCL2 family, contributing to the avoidance of cell death by acting as a regulator of apoptosis in some human malignancies[54]. In OS, MCL-1 expression is upregulated after chemotherapy, and high MCL-1 expression is associated with poor overall survival, increased recurrence rate, decreased sensitivity to MTX, and promotion of tumor proliferation[55]. In OS cells, MCL-1 is a direct target of miR-375; overexpression of miR-375 enhances the effects of cisplatin-induced DNA damage mediated by MCL-1[54]. Regorafenib is an oral type II multikinase inhibitor that inhibits the vascular endothelial growth factor receptor 1-3 (VEGFR1-3), platelet-derived growth factor receptors, fibroblast growth factor receptors (FGFR), tyrosine kinase receptor with immunoglobulin-like and EGFR-like domains 2 (TIE-2), and pathways involved in angiogenic and metastasis process. Sorafenib has been approved as a second-line treatment in OS, as discussed below[56]. MCL-1 is an essential survival factor for endothelial cells (EC) required for blood vessel production during angiogenesis. Deletion of MCL-1 in EC cells resulted in a dose-dependent increase in EC apoptosis in the angiogenic vasculature and reduced vessel density. Inhibition of vascular endothelial growth factor A (VEGF-A) may cause EC apoptosis[57]. The role of MCL-1 in regorafenib resistance has been evaluated in colorectal cancer. Regorafenib-resistant cells are deficient in MCL-1 degradation, MCL-1 is associated with PUMA and inhibits apoptosis, and MCL-1 inhibitor overcomes acquired resistance to regorafenib by liberating PUMA from MCL-1, restoring apoptosis. Thus, inhibition of MCL-1 also seems to overcome the resistance to regorafenib[58].

MCL-1 appears to be a new therapeutic target in OS. Inhibitors of MCL-1 can be used in different settings, e.g., to restore sensitivity to chemotherapy or anti-angiogenic resistance or in combination with other agents to increase therapeutic efficacy. A phase 1 study of a MCL-1 inhibitor in solid tumors, including sarcomas, is currently ongoing[59].

SIGNAL TRANSDUCTION PATHWAYS

Human epidermal growth factor receptor family and OS

Since the 1990s, the expression of human epidermal growth factor receptor 2 (HER2) has been reported in OS primary tumors and metastases[60]. HER2 is overexpressed in approximately 32%-45% of OS samples. Some studies have associated HER2 with worse event-free survival and metastasis-free survival; they have also correlated the overexpression of HER2 with poor response to chemotherapy[60]. Based on this evidence, strategies directed against HER2 to increase the survival of patients with OS HER-positive tumors have emerged. The use of trastuzumab in combination with cytotoxic chemotherapy was investigated in a phase 2 trial of 96 patients newly diagnosed with metastatic OS; of those, 41 had tumors expressing HER2. There was no difference in event-free survival or overall survival between the HER2-positive and HER-negative groups; trastuzumab has not been tested in other randomized trials[61]. The location of HER2 in OS probably contributes to the failure of treatment with monoclonal antibodies; however, there is a need to develop other intracellular inhibitors for the blockage of this pathway. Overexpression of HER2 has been involved in mechanisms of resistance to cisplatin mediated by phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) activation. The basal activity of PI3K/AKT1 upregulates cyclin-dependent kinase inhibitor 1A (CDKN1A; also termed p21), promoting cell cycle arrest and leading to time for DNA repair; additionally, HER2 overexpression mediates the nuclear exclusion of p21 and activation of PI3K/AKT, favoring cell proliferation. Both aforementioned mechanisms have been described in resistance to cisplatin[34,62]. In addition, overexpression of HER2 has been associated with resistance to cisplatin in clinical settings[62]. The inhibition of this pathway could be explored in second-line therapy to overcome this resistance in patients with OS. Now, a new drug is being tested in an ongoing phase 2 trial (identifier: NCT04616560) for the treatment of HER2-positive patients with recurrent OS. Trastuzumab-deruxtecan is a conjugated antibody-drug composed of a humanized monoclonal antibody specifically targeting HER2 and a potent topoisomerase I inhibitor as the cytotoxic drug. Trastuzumab attaches to HER2-positive cancer cells. HER-2 receptor works as a target for trastuzumab to deliver deruxtecan into OS cells[63] [Figure 2].

Other members of the HER family have been investigated in OS. A moderate-to-high expression of epidermal growth factor receptor (EGFR) is present in 50%-90% of OS samples, and the expression of EGFR is associated with a higher rate of metastasis, risk of recurrence, and resistance to chemotherapy[64,65]. Treatments with EGFR inhibitors, such as gefitinib, inhibited OS cell growth and sensitized EGFR-expressing cells to chemotherapy, DOX, and MTX[65]. The combination of gefitinib with DOX and MTX also showed a synergistic impact on cell proliferation and apoptosis[66]. Furthermore, the monoclonal antibody cetuximab decreased OS cell motility via PI3K/AKT/mitogen-activated protein kinase (PI3K/AKT/MAPK) signaling[67]. Canertinib (CI-1033), EGFR, and HER2 inhibitor induced apoptosis and decreased EGFR and HER2 phosphorylation in OS cells[68]. ZD6474, a dual tyrosine kinase inhibitor (TKI) of EGFR and vascular endothelial growth factor receptor (VEGFR), inhibited OS cell growth, induced cell cycle arrest, and promoted apoptosis and tumor growth in nude mice[69]. HER4 expression is associated with low probabilities of survival and metastasis-free survival. Knockdown of HER4 decreased cell viability upon treatment with MTX and DOX and increased apoptosis of OS cells based on cleaved PARP, suggesting that downregulation of HER4 increases the sensitivity of OS cells to chemotherapeutic drugs; HER4 also interacts with NDGR1 (N-myc downstream regulated gene), which contributes to cell growth and survival in OS cells[70]. Afatinib is a TKI that selectively blocks the signaling of homodimers and heterodimers formed by EGFR, HER2, HER3, and HER4 in OS cell lines[71]. It has been observed that afatinib inhibits the proliferation, migration, and invasion of non-metastatic and metastatic OS cell lines. Moreover, it decreases the phosphorylation of HER2/EGFR receptors and downstream molecules AKT and extracellular signal-regulated kinase 1/2 (ERK1/2)[72]. Using sarcospheres from highly metastatic human OS cell lines, Collier *et al.* revealed that afatinib also has therapeutic potential with this technology[73]. Recently, a meta-analysis of the gene expression signature of primary OS samples using the Gene Expression Omnibus microarrays series was performed to establish the OS gene signature. The Characteristic Direction Signature Search

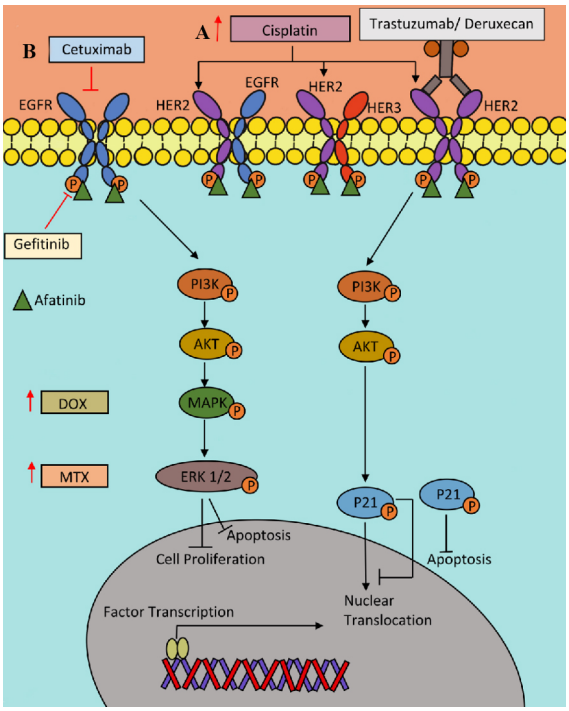

Figure 2. Role of Family HER Pathway in osteosarcoma chemotherapy resistance. (A) Cisplatin resistance is associated with HER2 overexpression, PI3K/AKT activation and promotion p21 nuclear exclusion, favoring cell cycle arrest and proliferation. (B) Anti-EGFR therapy such as cetuximab and gefitinib sensitized osteosarcoma cells to DOX and MTX. (C) Afatinib a pan-HER Family inhibitor have an inhibition effect in osteosarcoma cell proliferation, migration an invasion. (D) Trastuzumab deruxtecan is an antibody-drug conjugated composed by anti-HER2 humanized monoclonal antibody and a topoisomerase I inhibitor as cytotoxic drug, that is now been tasted in clinical trials. AKT: Protein kinase B; DOX: doxorubicin; EGFR: epidermal growth factor receptor; ERK: extracellular signal-regulated kinase; HER: human epidermal growth factor; MTX: methotrexate; PI3K/AKT: phosphoinositide 3-kinase.

Engine was used to identify the most appropriate molecules for reversing this gene expression signature in OS and propose new potential drugs. The authors found 266 genes (98 upregulated and 168 downregulated) in OS, and afatinib appeared as one of the top molecules for reversing this signature[74]. Afatinib is currently being tested in a phase 2 trial in pediatric tumors, including rhabdomyosarcomas and those with HER deregulation recurrent/refractory disease after receiving at least one prior standard treatment regimen (identifier: NCT02372006)[75]. This trial will let us see the activity of afatinib in sarcomas and patients with dysregulated HER pathway; its potential role in OS has to be evaluated directly in future clinical trials.

PI3K, mechanistic target of rapamycin, AKT, and microtubule affinity-regulating kinase 2 pathways in OS

The PI3K/AKT signaling pathway is involved in cell survival and the RAS, RAF, and ERK/MAPK pathways, which mediate tumor proliferation and growth and are downstream of cell-surface receptors in OS[76]. One of the targets of this pathway is MARK2. MARK2 is a serine/threonine kinase implicated in microtubule-associated protein phosphorylation and cell cycle regulation. This protein is associated with neurological disorders, cell polarization, intracellular transport, and migration. Overexpression of MARK2 is associated with poor prognosis in patients with OS. Some mechanisms by which MARK2 may increase the resistance to cisplatin in OS have been proposed. For example, MARK2 may mediate resistance to cisplatin in OS by inhibiting apoptosis through the expression of BCL2. Another mechanism is the regulation of P-gp expression mediated by MARK2. The expression of P-gp and MARK2 in the MG-63 and MNNG/HOS OS cell lines is upregulated compared with that observed in osteoblasts. Silencing of MARK2 in OS cells also decreased P-gp expression, suggesting a relationship between these two proteins. In the case of cisplatin-

resistant cells, following blockage of MARK2, P-gp expression is reduced and sensitivity to this chemotherapeutic agent is improved. This regulation may be mediated by the activation of the PI3K/AKT/nuclear factor-κB (PI3K/AKT/NF-κB) pathway[77]. MARK2 appears to regulate the DNA damage repair dependent on NHEJ mediated by DNA-dependent protein kinase (DNA-PK) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs). High levels of DNA-PKcs were correlated with poor prognosis as well as an increased risk of recurrence and metastasis in patients with OS. Furthermore, the expression of this catalytic subunit was increased in MG63 cells treated with cisplatin and etoposide[78]; the expression of DNA-PKcs in cisplatin-resistant MG-63 OS cells appears to be regulated by MARK2 via the PI3K/AKT/mTOR pathway. In this pathway, the protein most strongly associated with DNA damage repair is AKT; it has been reported that DNA damage activates AKT. In addition, it appears that AKT phosphorylation on S473 is dependent on DNA-PK; AKT1 forms a complex with DNA-PKcs, resulting in the activation and auto-phosphorylation of the S2056 of DNA-PKcs[79]. Another important protein is mTOR, a key regulator of the PI3K/AKT pathway; overactivation of mTOR is associated with resistance to cisplatin. The PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways interact at different levels. RAS activates PI3K by interacting with its catalytic subunit, and ERK2 phosphorylates TSC complex subunit 2 (TSC2), suppressing its function and promoting the activation of mechanistic target of rapamycin complex 1 (mTORC1). Downstream of RAS/ERK is the 90 kDa ribosomal protein S6 (RPS6); this kinase phosphorylates TSC2 at Ser1798 and inactivates its tumor suppression function, allowing mTORC1 signaling[79]. With regard to MAPK, a special activation was demonstrated in high-grade OS specimens, showing inhibition of ERK1/2 phosphorylation and increased expression of proapoptotic proteins (e.g., BAX) that induce apoptosis in OS cells; the inhibition of ERK1/2 also increased the sensitivity of OS cells to DOX[80]. A phase 2 trial of sorafenib in combination with everolimus (an inhibitor of mTOR) in OS was designed to overcome the resistance to sorafenib. Sorafenib inhibits the activity of the mTORC1 complex but activates the mTORC2 complex and promotes tumor progression. Preclinical studies have shown that everolimus effectively overcomes this resistance mechanism. The study was designed to include high-grade patients with OS who had progressed after receiving a MTX/DOX/cisplatin /IFOS chemotherapy regimen that included MTX, DOX, cisplatin, and/or IFO. Interestingly, the group of patients who expressed phosphorylated-ERK1/2 (p-ERK1/2) and p-RPS6 presented a greater response to the combination of sorafenib and everolimus than the negative expression group (clinical trial identifier: NCT01804374)[81]. These findings suggest that, after chemotherapy, the PI3K/AKT pathway appears to be active in a subgroup of patients. Therefore, establishing the activation of the PI3K pathway prior to treatment may assist physicians in selecting patients who would benefit from treatment with an mTOR inhibitor alone or in combination with other agents. Further clinical trials based on molecular profiles are warranted to explore the combination of a mTOR inhibitor with a multikinase inhibitor with a different profile that may include the RAS/MEK/ERK pathway as targets.

TME AND ANGIOGENESIS

OS therapy has only been partially effective, possibly due to the existence of compensatory pathways, the inherently heterogeneous nature of sarcomas, and the complex interaction with the TME. Multiple intermingled cell types, such as osteoblasts, osteoclasts, fibroblasts, immune cells, macrophages, vascular cells, mesenchymal stem cells (MSC), and hematologic progenitor cells, coexist in stroma bone[82]. The heterogeneity observed in different tumor cell subpopulations is modulated by different mechanisms, including the extracellular matrix (ECM) and its interactions with intracellular and extracellular elements, metabolites, oxygen tension, pH, *etc*. For instance, an alteration of the RB pathway is sufficient to induce anchorage-independent growth of these tumors; additionally, clonal evolution is dependent on the environment (hypoxia and immune infiltrate) and can result in resistance or tolerance, while microenvironment communications (e.g., angiogenesis, immune stimulation, and initiation of signal

transduction) complicate this tumoral scenario[83,84]. Crenn *et al.* evaluated the histological response to chemotherapy (i.e., IFO, cisplatin, and DOX) in murine MOS-J cell models, mimicking various microenvironments by injecting tumor cells into subcutaneous, intramuscular paratibial, and intra-osseous sites[85]. A higher response to DOX was observed in the intra-osseous model compared with the intramuscular model in terms of tumor growth and necrosis, suggesting that a more vascularized intra-osseous ambient favors drug action[25,85]. This conclusion was based on the premise that bisphosphonates inhibit osteoclast bone resorption, reduce therapy-induced bone loss, and improve anticancer activity by inhibiting angiogenesis, invasion, tumor cell adhesion, and enhancing immunity. Additionally, in preclinical murine models, zoledronate exerted antitumor effects on OS cells, reduced tumor growth, reduced lung metastasis, and improved survival. Based on this evidence, a phase 3 trial (OS2006) evaluated the addition of zoledronate to conventional chemotherapy; unfortunately, this therapeutic strategy failed to improve the pathological preoperative response to chemotherapy and clinical outcomes. The authors explained this negative result as the effect of zoledronate on immunological parameters such as NK-cell expansion, macrophage depletion, or polarization may affect the bone microenvironment; the absence of benefit with zoledronate combined with chemotherapy may be related to a potential upregulation of RANK expression that promotes osteosarcoma pathogenesis by osteosarcoma cells[86,25,87].

Other members of TME are immune cells, which include cells of the innate and non-innate immune response. Osteosarcoma has several alterations in DNA repair that translates into mutation implicated in their carcinogenesis that may produce mutational antigens attractive to immune cells; however, similar to other sarcomas, tumor mutation burden (TMB) in OS is low. Nevertheless, the microenvironment tumor-associated inflammatory infiltrate is a strong prognostic indicator of response to therapy and overall survival. Osteosarcoma had consistently low expression of PD-L1 in studies and can be classified as a "cold tumor"; however, immune cells (e.g., CD8+ cytotoxic T-lymphocytes) are associated with a favorable prognosis in OS studies.

Based on this, the phase 2 PEMBROSARC trial evaluated the anti-programmed cell death 1 (anti-PD-1) pembrolizumab in combination with alternating cyclophosphamide in 17 patients with OS; only 13.3% of patients had stable disease at 6 months[88]; the median progression-free survival was 1.4 months and median overall survival was 5.6 months. This study showed interesting results; for example, none of the three patients with tumor shrinkage had an expression of PD-L1 on sarcoma or immune cells and only 12% of the cases were PD-L1 positive in this study[88]. One explanation could be the implication of other mechanisms implicated in immune response in OS, such as other members of TME and the tumor survival mechanism[89]. In the PEMBROSARC trial, the authors observed an increase in the kynurenine to tryptophan ratio in the third cycle of pembrolizumab compared to the first cycle. The kynurenine pathway requires tryptophan and IDO. The authors of this trial mentioned the inhibition of IDO in combination with pembrolizumab as a strategy to be explored in these tumors[90]. Tumor-derived exosomes can inhibit T cell and NK cell activity through different pathways, and T cell apoptosis favors immune surveillance escape and activation of bone marrow-derived suppressor cells (MDSC). Around 36% of patients with breast cancer have exosomes containing indolamine deoxygenase (IDO), which regulates antitumor immunity by depletion of tryptophan levels, promoting inflammatory microenvironment and angiogenesis[91,92]. As mentioned above, it can be related to immune checkpoint inhibitor response. Release of exosomes with soluble major histocompatibility complex I chain-related proteins (SMIC) and NKG2D (natural killer cells receptor) soluble receptors are implicated in the downregulation of NK cells and cytotoxic T lymphocytes[91,93]. Another possible pathway implicated in OS immune response is transforming growth factor-beta (TGF-β), which plays an important role in excluding T cells from the tumor microenvironment and, unfortunately, is present in high levels in OS patients, especially in the metastatic OS setting; thus, the

inhibition of this pathway may improve the antitumor effects of immunotherapy[91,94,95]. Combination therapy has emerged as a future strategy to enhance chemotherapy and immunotherapy; for example, in OS cells, DOX increases apoptosis in CD8+ T cells. However, this effect was reversed by the anti-PD-L1 antibody and the combination of the anti-PD-1 antibody and cisplatin inhibits tumor growth[70].

This finding indicates that exploration of other pathways in the TME for the treatment of OS is warranted. Tumor-associated macrophages play a pivotal role in the regulation of local immunity, angiogenesis, and tumor cell migration; they are divided into two principal macrophage populations (M1 and M2). In OS, M1 and M2 infiltrations are associated with better and worse outcomes, respectively. It has been observed that patients with infiltration of CD68+ cells in OS tissues typically exhibit a poor response to neoadjuvant chemotherapy; however, after treatment with chemotherapy, macrophages secrete interleukin-1β (IL-1β) and reduce the sensitivity of OS to cisplatin[96]. Macrophages promote angiogenesis and contribute to the development of resistance to chemotherapy; the deletion of vascular endothelial growth factor A (VEGFA) in macrophages leads to normalized vascular growth, reduces hypoxia, and increases sensitivity to cisplatin[97,98]. Apatinib, a selective tyrosine kinase inhibitor to VEGFR2, inhibits epithelial-mesenchymal transition (EMT) and PD-L1 expression by targeting STAT3 *in vitro* and *in vivo*[99]. Future strategies, such as the combination of immunotherapy, chemotherapy, and anti-angiogenesis therapy or more complex alternatives such as vaccines or modified immune cells, are being tested in osteosarcoma to overcome immune surveillance escape and chemotherapy resistance[91].

The ECM constitutes a three-dimensional acellular network of macromolecules that provide structural and biochemical support to cells, including malignant cells[100]. Moreover, the ECM is implicated in cell communication, migration, adhesion, proliferation, and differentiation. Components of the ECM (e.g., collagen, fibronectin, laminin, and proteoglycan) are implicated in OS cell growth, proliferation adhesion, invasion, metastasis, resistance to chemotherapy, and angiogenesis. A high expression of collagens, collagen triple helix repeat containing 1 (CTHRC1), and collagen type I alpha 1 chain (COL1A1) has been associated with shorter survival. Overexpression of collagen type III alpha 1 chain (COL3A1) may decrease apoptosis and promote resistance to MTX in OS cell lines[101]. Tumstatin is a 28 kDa protein fragment of COL4A3 (a non-collagenous domain of the alpha 3 chain in collagen IV) with an anti-angiogenic capacity that inhibits cell proliferation and induces apoptosis in OS cells[102-104]. Endostatin is a 20 kDa terminal-C fragment of collagen XVIII that inhibits angiogenesis by directly binding to both VEGFR1 and VGFR2. Endostatin is also associated with different surface integrins; it competes with the fibronectin pro-angiogenic ligand for binding to integrin a5β1 to disrupt cell migration, activates SRC and caveolin 1 (CAV1), disassembles focal adhesion fibers and actin stress fiber, and inhibits cell migration[101,105]. Both tumstatin and endostatin inhibit the phosphorylation of focal adhesion kinase (FAK) downstream of FAK; subsequently, tumstatin FAK inhibits the PI3K/AKT/mTOR/4EBP1 pathway downstream, resulting in the inhibition of endothelial protein synthesis. Endostatin inhibits the activation of the ERK1/p38 MAPK pathway that inhibits the migration of endothelial cells[75,106]. In preclinical models, the combination of recombinant human-endostatin (rh-endostatin) with DOX produced an important synergistic antitumor activity[107]. Rh-endostatin has been investigated in combination with conventional chemotherapy (i.e., MTX, DOX, and cisplatin) in patients recently diagnosed with OS; this strategy improved the clinical outcomes of chemotherapy, prolonging the 2- and 5-year event-free survival of 81% and 75% of patients, respectively, in the rh-endostatin group *vs.* 67% and 57% of patients, respectively, in the chemotherapy alone group; the relative risk in the rh-endostatin group was 0.49 (95%CI: 0.36-0.078, $P = 0.010$). Rh-endostatin reduced the 2- and 5-year distant metastasis-free survival in 82% and 79% of patients, respectively, in the combination group compared with 71% and 61% of patients, respectively, in the chemotherapy alone group; the relative risk of distant metastasis in the rh-endostatin group was 0.48 (95%CI: 0.30-0.76, $P = 0.014$)[108,109].

Interestingly, patients in the control group exhibited increased VEGF expression and microvascular density (MVD) after exposure to chemotherapy alone; however, patients in the rh-endostatin group showed reduced VEGF expression and MVD. Similar results have been observed in the neoadjuvant setting with DOX, cisplatin, MTX, and IFO, where rh-endostatin reduced VEGF expression and MVD, and improved distant metastasis-free survival and overall survival; these findings suggest again a protective effect in preventing lung metastasis[110]. This supports the rationale that early intervention with anti-angiogenic therapy in combination with conventional chemotherapy may reduce the risk of angiogenesis-dependent metastasis. Endostatin also improved clinical outcomes in patients with stage IV OS; in combination with chemotherapy, endostatin increased the progression-free survival (8.6 *vs.* 4.4 months) and the clinical benefit response (47.8% *vs.* 16.7%)[111].

Angiogenesis plays an important role in the development and progression of OS; tumor micro-vessel density and VEGF expression have been associated with the prognosis of OS[112,113]. Chemokines promote angiogenesis in OS cells mainly through the following two pathways[113]. Firstly, C-C motif chemokine ligand 3 (CCL3) enhances VGEFA expression, facilitates progenitor cell migration, and promotes tube formation by downregulating the expression of microRNA (miRNA) 374b via JUN N-terminal kinase/ERK (JNK/ERK) and p38[114]. Secondly, CCL5 increases VEGF expression and promotes its pathway by the protein kinase C/cellular-SRC/hypoxia-inducible factor 1 alpha (PKCδ/c-SRC/HIF-1α) signaling pathway[115]. Higher expression of VEGFA is present in OS cells resistant to anoikis via the SRC, JNK, and ERK pathways. The use of a SRC inhibitor reduced the expression of VEGFA and angiogenesis via the inhibition of JNK and ERK activity. The overexpression of p-SRC and VEGFA is also correlated with metastatic potential in human tissues[116]. Relaxin is a peptide family belonging to the insulin superfamily that promotes the tumor growth, invasion, and angiogenesis of Sao-2 cells via AKT/VEGF. Relaxin H2 (RLN2) confers migratory and invasive capabilities, as well as resistance to cisplatin by modeling the AKT/NF-κB in U2OS and MG63 cells[117]. In patients with OS, genes of the VEGF pathway are amplified; the most frequent copy-number aberration is the amplification at 6p12-21 that involves *VEGFA* (27%), and a subset of tumors had amplifications in 4q11-12, including platelet-derived growth factor receptor A (*PDGFRA*) and kinase insert domain receptor (KDR) (18%). These findings suggest angiogenesis as a target in OS[46]. The activation of this pathway in OS affects survival outcomes. Notably, the receptors of the VEGFR pathway, VEGFR2 and VEGFR3, are associated with worse survival in patients with OS, while the ligand VEGFB is associated with poor histologic response to chemotherapy[118]. High expression of VEGFR3 and PDGFRB is associated with high-grade OS tumors[112,118]. Recently, a meta-analysis showed that high levels of VEGF are also associated with advanced tumor stage and metastasis, with negative consequences in terms of survival; high VEGF expression is implicated in worse disease-free survival (odds ratio = 0.25, 95%CI: 0.11-0.58, $P = 0.001$) and overall survival (odds ratio = 0.22, 95%CI: 0.13-0.35, $P \leq 0.001$)[119]. These new pathways described in OS lead to the evaluation of different multikinase drugs in preclinical and clinical studies. Monotherapy with multikinase drugs has been explored in patients with OS who previously received chemotherapy. Table 1 presents the principal targeted therapies involved in signaling pathways in osteosarcoma, principally the angiogenic pathway, studied in phase 2 and observational trials as second-line therapy for OS.

FAK and SRC inhibitors to overcome resistance to chemotherapy

The signaling of integrins includes SRC AKT-ERK and FAK (a non-receptor cytoplasmic protein tyrosine kinase)[128]. The inhibition of SRC in murine models inhibited tumor growth and decreased the metastatic potential of OS cells. Moreover, this inhibition overcame the resistance to DOX and induced apoptosis in chondrosarcoma cells[129,130]. This approach has been evaluated in OS in the clinical setting; monotherapy with saracatinib (AZD530) was well tolerated in patients with OS, showing a median progression-free survival of 19.4 months *vs.* 8.6 months in the placebo group ($P = 0.47$). The investigators concluded that the

Table 1. Principal targeted therapies involved in the signaling pathways in osteosarcoma

Agent	Signaling pathway	Pediatric dose	Adult dose	RR%	4-month PFS (%) (95%CI)	6-month PFS (%) (95%CI)	Median PFS (months) (95%CI)	Reference
Phase 2 studies								
Apatinib[a]	RET, VEGFR1,2	500 mg/day[b]	750 mg/day[b]	43	57 (39-71)	37 (21-52)	4.5 (3.5-6.3)	[120]
Cabozantinib	KIT, MET, RET, VEGFR1,2,3	40 mg/m^2 /day	60 mg/day	12	71 (55-83)	52 (36-66)	6.7 (5.4-7.9)	[121]
Lenvatinib	RET, VEGFR1,2,3	14 mg/m^2 /day	14 mg/m^2 /day	7	33		3.4 (NR)	[122]
Regorafenib	KIT, RET, PDGFRB, VEGFR1,2,3	160 mg/day[c]	160 mg/day[c]	8	44.4	45	3.6 (2-7.6)	[123,124]
Sorafenib	KIT, RET, VEGFR1,2,3, PDGFRA,B	400 mg b.i.d.	400 mg b.i.d.	29	46 (28-63)	9	4 (2-5)	[125]
Combinations								
Sorafenib/Everolimus	PI3K/AKT, mTORC1,2		S: 400-600 mg/day E: 2.5-5 mg/day		45			[81]
Observational studies								
Pazopanib	VEGFR, PDGFR, KIT, FGFR		400-800 mg/day	68			5.5[d] (2.7-7.7)	[126]

Adapted from Just *et al.*[127]. AKT: Protein kinase B; b.i.d.: twice daily; BSA: body surface area; CI: confidence interval; E: everolimus; FGFR: fibroblast growth factor receptor; KIT: stem cell factor receptor; mTORC: mammalian target of rapamycin complex; NR: not reported; PDGFR: platelet-derived growth factor receptor; PFS: progression-free survival; PI3K: phosphoinositide 3-kinase; RET: rearranged during transfection; RR: response rate = complete + partial responses; S: sorafenib; VEGFR: vascular endothelial growth factor receptor. [a]Apatinib is not approved by the US Food and Drug Administration. [b]Dose for patients with BSA < 1.5: 500 mg/day; dose for patients with BSA > 1.5: 750 mg/day. [c]Drug is administered daily for 21 days in 28-day cycles. [d]Study reported a 10-week PFS.

results of SRC inhibition alone are insufficient to suppress metastatic progression[131]. Dasatinib combined with ceritinib, an off-target inhibitor of insulin-like growth factor 1 receptor (IGF1R), was tested in one patient; the treatment was well tolerated and showed limited toxicity. Additionally, patient tissue analysis revealed high necrosis and extensive infiltration of macrophages, suggesting that this combination is a promising strategy[132]. FAK is downstream of SRC, and tyrosine 397 is the major site of autophosphorylation in the FAK catalytic domain. FAK is related to several tumor processes, such as vascular and microenvironment regulation, proliferation, motility, invasion, and survival[133]. Integrin-β1 (ITGβ1) activates FAK and ERK in MG63 OS cells, and plays an important role in the proliferation and differentiation process of osteoblasts[134]. High expression levels of FAK are associated with advanced disease and recurrence in patients with OS, rendering it a potential biomarker[135,136]. The levels of total FAK and p-FAK-Y397 have been evaluated in OS tissues. Overexpression of FAK was detected in OS, and overexpression of thep-FAK-Y387 was correlated with poor histologic response to chemotherapy (i.e., MTX, DOX, cisplatin, etoposide, cyclophosphamide, IFO, and carboplatin)[135]. In xenograft models, the decrease in FAK expression using FAK inhibitors impaired OS cell proliferation and colony formation and reduced tumor growth[136]. Platinum-resistant tumorspheres can acquire a dependence on FAK for growth, and the combination of a FAK inhibitor with platinum overcomes resistance to cisplatin[137]. Ongoing clinical trials evaluate the safety and efficacy of FAK inhibitors in different solid tumors[138-145]. Figure 3 shows the signal pathways involved in OS.

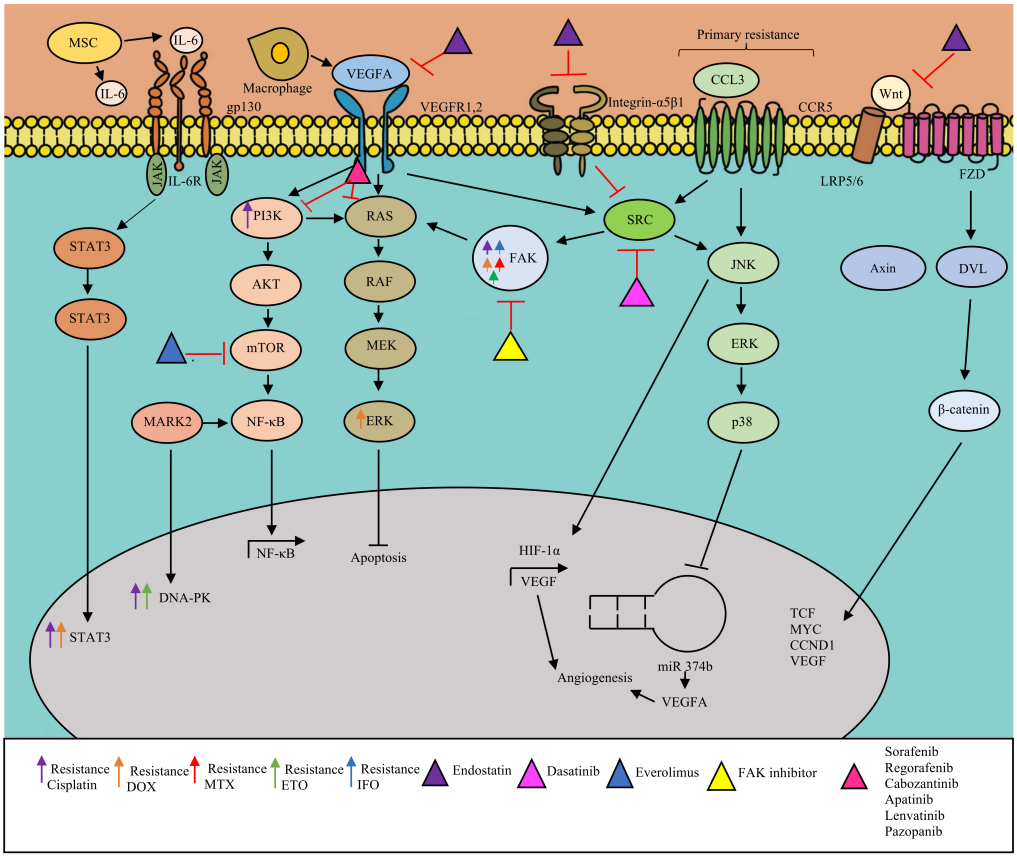

Figure 3. Angiogenesis and osteosarcoma microenvironment. (A) Mesenchymal stem cells (MSCs) produce IL-6 and STAT3 pathway activation promoting cisplatin and DOX resistance. (B) Macrophages promotes angiogenesis by VEGFA production in osteosarcoma. (C) VEGFA ligand binds to VEGFR activating angiogenesis pathway, throw PI3K/AKT/mTOR or Ras. Some anti-angiogenesis tyrosine kinase inhibitors alone or in combination with mTOR inhibitors are approved in second line in osteosarcoma. (D) Integrin pathway actives SRC and FAK promoting angiogenesis and apoptosis inhibition. (E) Chemokine (C-C motif) ligand 3 (CCL3) binding to G-protein coupled C-C chemokine receptor 5(CCR5) promoting VEGFA expression by downregulation of miR-374b, activation of JNK/ERK/p38 and hypoxia-inducible factor (HIF) in human osteosarcoma cells. (F) The canonical Wnt/β- catenin pathway contributes to chemotherapy resistance and osteosarcoma progression. WNT actives the Frizzled (FZD) and low-density lipoprotein receptor 5/6 (LRP5/6) binding disheveled (DVL) and Axin protein complex release of β-catenin and lead the translocation of β-catenin to the nucleus to activates genes active in chemoresistance. (G) Endostatin inhibit the activity of integrin, VEGFR and WNT pathways. AKT: Protein kinase B; CCD1: CyclinD1; DOX: doxorubicin; DNA-PK: DNA-dependent protein kinase, ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; gp130: glycoprotein 130; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; IL-: interleukine-6; IL-6R: IL-6 receptor; JAK: Janus Kinase; JNK: JUN N-terminal kinase; MARK2: microtubule affinity-regulating kinase 2; MYC: myc proto-oncogene; NF-κB: nuclear factor-κB; SRC: SRC protein kinase; STAT3: signal transducer and activator of transcription 3; VEGF: vascular epidermal growth factor; VEGFR: vascular epidermal growth factor receptor.

TME hypoxic condition: the roles of acidosis, lactate, and adenosine in OS and therapy resistance

Angiogenesis implies newly formed microvessels with altered morphology compared with normal vessels. These abnormalities work as a biophysical barrier to the delivery of oxygen, nutriments, and antitumor therapies to a solid tumor. Oxygen delivery difficulties favor metabolic changes in TME characterized by hypoxic conditions, extracellular acidosis, substantial elevated adenosine and lactate concentrations, and nutrient deprivation[146]. TME hypoxic condition contributes to genetic instability, intratumorally heterogeneity, malignant progression, tumor stem cell maintenance, angiogenesis, development of treatment resistance, and metabolic reprogramming dependent on HIF-1α phenotype. These stress conditions that activate HIF-1α may serve as major drivers for recruitment, activation, polarization, and expansion of immune-suppressive stromal cells and affect the antitumor activity of the innate and adaptive immune system, as well as cancer immunotherapy. Hypoxia-/HIF-driven factors include generation and

accumulation in the extracellular space of adenosine, extracellular acidosis, and favor overexpression of VEGF and activation of VEGFR related to immune suppression and anti-angiogenic therapy response[146,147]. Metabolic reprograming induced by hypoxia/ HIF-1α is characterized by glycolytic enzyme lactate dehydrogenase A (LDH-A) and accelerated glycolysis (Warburg effect) that contribute to lactate accumulation, affecting tumor T cell infiltration and cytokine productions, inhibits the NK and CD8+T cells cytotoxic activity and favors MDSCs infiltration. Tumor extracellular acidosis depends on: (A) the Warburg effect or upregulation/acceleration of glycolysis characterized by an intensive conversion of glucose to lactic acid and insufficient adenosine triphosphate (ATP) production to favor very fast energy supply; (B) increase in glutaminolysis; (C) ketogenesis; (D) increased ATP-hydrolysis, i.e., hydration of CO_2-derived from oxidative metabolism and pentose phosphate pathway; and (F) bicarbonate depletion in TME[146]. Acidosis (extracellular pH 6.8) has immune-suppressive actions such as inhibition of the proliferative and cytotoxic activity of NK and CD8+ T cells, secretion of IFN-, and reducing the expression of T cell receptors[148,149]. Acid tumor microenvironment may be related to resistance in osteosarcoma. Extracellular pH in P-gp-negative cell lines reduced sensibility to DOX, and the combination of DOX with the proton pump inhibitor omeprazole enhanced its cytotoxicity capacity and reduced tumor volume in OS animal models; similarly, the pH gradient rendered in OS cells increased response to cisplatin and MTX[150,151]. Hypoxic stress induces cancer cells' ATP to release through pannexin 1 PANX-1 channels and exocytosis of adenosine and promotes the accumulation of adenosine in the extracellular space of hypoxic tumors. Adenosine attenuates the activity of T cells, NK cells, and dendritic cells and enhances the suppressive capacity of T regulatory cells (Tregs) and MDSCs. Hypoxia increases the accumulation of extracellular adenosine mainly produced by enzymatic ATP catabolism; adenosine induces the expression of adenosine receptors in tumor cells, promoting growth, survival, and metastasis[152]. Non-regulated release of adenosine occurs from dying and damaged cells, whereas the active release involves exocytotic granules, plasma membrane-derived macrovesicles, specific ATP-binding cassette (ABC) transporters and membrane channels (connexin hemichannels and PANX1), calcium homeostasis modulator 1 (CALHM1), volume-regulated anion channels (VRACs), and maxi-anion channels (MACs). The extracellular ATP (eATP) activity is via P2 purinergic receptors, P2X7R[153]. TME extracellular ATP is degraded by different ectonucleotidases, principally CD39 and CD73: the ectonucleoside triphosphate diphosphohydrolase CD39 hydrolyzes ATP to ADP and AMP, while the ecto-5'-nucleotidase (CD73) hydrolyzes AMP to adenosine. CD39 is expressed in dendritic cells, tumor-infiltrating Th17 lymphocytes, and M2 macrophages. CD73 is expressed in lymphocytes T and B, stromal cells, and dendritic cells[152,153]; it is expressed in Treg and in higher levels on anergic CD4+ T cells, preserving self-tolerance in healthy individuals. CD73 has a role as an immune-inhibitory checkpoint molecule, contributing to tumor infiltration of regulatory immune cells such as Treg, MDSCs, or DCs, favoring an immunosuppressive microenvironment[154]. CD73 is overexpressed in many tumors and promotes cell migration, invasion, and chemotherapy resistance[155]. In human osteosarcoma cell lines, miR-16 indirectly downregulates CD73 expression and inhibits the expression of transcription factors SMAD3 and SMAD4, both implicated in CD73 expression[154,156]. In hypoxic TME, hypoxia induces CD73 expression via HIF-1α regulating EMT and promotes lung metastasis in triple-negative breast cancer[157]. Different combination strategies of CD73 pharmacological inhibition with A2BR antagonist, chemotherapy and radiotherapy, anti-PD1/PD-L1 therapy, and anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) improve cancer therapies[152]; Durvalumab (monoclonal antibody anti-PD-L1) and oleclumab (anti-CD73 monoclonal antibody) are being tested in NCT04668300 trial; this study includes osteosarcoma patients. The CD73 activity is mediated by P1 purinergic receptors (P1Rs), G-protein-coupled receptors divided into 4 subtypes: A1R, A2AR, A2BR, and A3R[158]. A1 receptor is involved in proinflammatory and anti-inflammatory processes, especially in the neurological system. A2AR protects host tissue from destruction secondary to an over-reaction of the immune response. A2AR activation inhibits DC4+ and CD8+ T-cell function and selectively inhibits proinflammatory cytokine expression, promoting the upregulation of PD-1. CTLA-4 promotes T-cell tolerance and prevents the development of IL-17,

promoting the development of Foxp3+ and LAG3+ regulatory T-cells. Adenosine acting via A2AR inhibits dendritic cell function. Since chemotherapy and radiation increase eATP, a concomitant administration of A2AR antagonists during chemotherapy or radiation might lead to the expansion of tumor T cells and prevent Treg cell induction. These drugs are being explored in a clinical trial with anti-PD1-PD-L1 therapy[159]. A2BR is related to pathophysiological conditions associated with adenosine releases, such as ischemia and tumor hypoxia. A2BR regulates several functions including vascular tone, cytokine release, and angiogenesis. The A2B receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation *in vivo*[160]. ATP release channels are also involved in osteosarcoma. Connexin 43 (Cx43), an implicated gap junction-mediated intercellular communication, is involved in proliferation suppression of human osteosarcoma U2OS cells by inhibition of the cell cycle transition, attributed to significant accumulation of hypophosphorylated RB protein that secondarily decreases kinase activities of CDK2 and -4. Cx43 seems to inhibit U2OS cells by increasing the levels of p27 protein via post-transcriptional regulatory mechanisms[161]. Cx43 is regulated by small ubiquitin-like modifiers (SUMOs); SUMO-conjugating enzyme UBc9 protein is overexpressed in osteosarcoma. Silencing UBc9 by siRNA inhibits osteosarcoma cell proliferation[162]. ALMB-0168, a humanized monoclonal antibody that specifically binds to the extracellular domain of Cx43 and activates the Cx43 hemichannels in osteocytes, enhances the activation of Cx43 hemichannels in both cultured osteocytes and mice osteocytes and promotes ATP release. ALMB-0168 reduces bone cancer growth in murine models WT Cx43; this drug also increases levels of cytotoxic lymphocytes (CD3/CD8+) and helpers (CD3/CD4+), increases the survival rate, and reduces tumor metastasis[163]. Another adenosine receptor, P2X7, is highly expressed in osteosarcoma tissues. The OSc receptor promotes the growth and metastasis of human HOS/MNNG cells via PI2K7AKTGSK3β/β and mTOR/HIF/VEGF signaling. Nevertheless, eATP increases plasma membrane permeability for cytotoxic molecules such as doxorubicin by opening P2X7R pores[164]. P2X7RA and -B are present in osteosarcoma tissue, and P2X7RB positive tumors show increased cell density depending on TME[165]. Shock wave-induced ATP release forms osteosarcoma U2OS cells and promotes cellular uptake and cytotoxicity of methotrexate by altering cell membrane permeability in a P2X7 receptor-dependent manner[166]. Adenosine, its receptors, eATP, and its transport channels are implicated in diverse pathways of osteosarcoma carcinogenesis, which makes their use attractive for managing this disease and reversing resistance mechanisms.

Nanoparticles

The tumor microenvironment is pivotal to drug delivery in solid tumors; the different components of TME act as barriers, limit drug accumulation, and induce drug resistance. Nanoparticles are a novel tool against cancer; they accumulate passively within solid tumors via pores and fenestration of tumoral blood vessels, reaching the tumoral zone. Then, they are able to penetrate deeper into the solid tumor, where they could have high therapeutic efficacy, facing elevated interstitial fluid pressure and denser extracellular matrices. Several strategies have emerged to overcome these difficulties: surface penetrating peptides combined with magnetic field guidance, proteolytic enzymes prior to nanocarrier treatment, polymeric nanocapsules to preserve their activity for longer times, gold NPs, liposomes micelles, and micelleplexes have been tested in osteosarcoma[167,168]. Villegas *et al.* designed an enzyme nanocapsule attached to the surface of mesoporous silica nanoparticles as a nanocarrier model and observed higher penetrance of the nanoparticles within 3D collagen matrices of HOS OSc[169]. Other proposed nanoparticles include biogenic calcium carbonate with better biocompatibility, slow biodegradability, pH-sensitivity, and osteoconductivity. Specific carriers or ligands with drugs include bisphosphonates (BP), N-(2-hydroxypropyl) methacrylamide (HPMA), and tetracycline (TC), as they have potential bone targeting and are ideal for treating metastatic cancer due to their high affinity towards hydroxyapatite (HA)[168]. Preclinical studies using *in vitro* and *in vivo* osteosarcoma models show efficacy using thermo-sensitive hydrogel conjugated with methotrexate and alendronate, a microparticle delivery system loaded with cerium dioxide (CeO_2) nanoparticles and

doxorubicin. The developed pH-sensitive microparticles were combined with doxorubicin, liposomes of doxorubicin, lipopolymer encapsulating CRISP/Cas9 plasmids encoding VEGFA gRNA and Cas9, and more complex nanoparticles used in gene therapy, such as micelleplexes loaded with miR-145[168]. In the context of eATP described above, different strategies using this technology are used to prevent fast eATP degradation including highly biocompatible and biodegradable albumin nanoparticles loaded with ATP release[170], a pH-sensitive nanoplatform made up of chitosan (Cs) and mesoporous HA to deliver ATP to tumor cells[171].

MIRNA-MODULATED DRUG RESISTANCE IN OS

The miRNAs are small non-coding RNAs (length: 18-25 nucleotides) that repress translation and cleave mRNA by base pairing with the 3′-untranslated region of target genes. They have the potential to regulate several critical biological processes, including the differentiation, progression, apoptosis, and proliferation of tumor cells. It has been estimated that there are up to 1000 miRNAs in the human genome. More than 30% of the human genome is regulated by miRNAs that simultaneously target multiple genes; recent differences in miRNA expression profiles detected between cancer cells and their normal counterparts revealed that miRNAs are involved in the pathogenesis of cancer[172]. In recent years, in-depth miRNA research has validated the involvement of miRNAs in OS drug resistance, tumor initiation, and progression These oncogenic or tumor suppressor miRNAs play a role in sensitivity to chemotherapy through several mechanisms, including DNA damage response, apoptosis evasion, autophagy induction, tumor stem cell activation, and alteration of signaling pathways. Maire *et al*. performed miRNA expression profiling for 723 human miRNAs in seven OS tumors; they identified 38 miRNAs differentially expressed by ≥ 10-fold (28 and 10 were downregulated and upregulated, respectively)[172]. These miRNAs are involved in intracellular signaling pathways associated with drug resistance, proliferation, and metastasis in OS, including the Notch, RAS/p21, MAPK, Wnt, and Jun/FOS pathways[172]. It was recently shown that the upregulated miR-124 enhances the cellular response to various DNA-damaging drugs by binding to the 3′-untranslated region of the ATM interactor (ATMIN) and PARP1 mRNAs in U2OS cells[173,174].

Different miRNAs have been identified as direct targets of p53, which are closely associated with drug resistance and progression in OS. Among them, members of the highly conserved miR-34 family (miR-34a, -34b, and -34c) are important components of the p53 tumor suppressor pathway. It has been observed that the expression of these miRNAs is induced by p53 in response to DNA damage or oncogenic stress[175]. He *et al*. reported that the miR-34 family induced G1 arrest and apoptosis in OS cells through its targets CDK6, E2F transcription factor 3 (E2F3), cyclin E2 (CCNE2), and BCL2 in a p53-dependent manner[176]. Additionally, it has been observed that the loss of miR-31 is associated with defects in the p53 pathway, and the overexpression of miR-31 significantly inhibits the proliferation of OS cell lines[177].

In OS cells, miR-513a-5p suppressed the expression of APE-1, rendering tumor cells radiosensitive[178]. The use of APE-1-targeted small interfering RNA (siRNA) (i.e., pSilenceAPE-1) sensitized OS cells and tumors xenografts to the anti-angiogenic endostatin, while miR-765 downregulated APE-1 and sensitized OS cells to cisplatin. Therefore, targeting APE1 with miRNA or siRNA may be a treatment option for overcoming drug resistance in OS[179].

miR-138 is the most recently discovered miRNA involved in resistance to cisplatin, which shows lower expression in OS tissue than in normal tissue. This decrease may be related to its tumor suppressor capacity. When the levels of miR-138 are restored, there is a marked inhibition of cell proliferation and invasion, as well as increased sensitivity to cisplatin. It has been observed that this change in sensitivity can be partially abolished by overexpression of the enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*)

gene, which can block the activity of caspase 3 (CASP3), a critical enzyme for apoptosis. Therefore, *EZH2* is the specific target gene for miR-138, and this miRNA acts as a tumor suppressor in OS by enhancing the sensitivity to cisplatin[180]. Recent studies revealed that miR-367 executes several functions in tumors and acts as an onco-miRNA in OS. Overexpression of miR-367 is associated with strong resistance to treatment with DOX. This effect is mediated by the decreased expression of the Kruppel-like factor 4 (KLF4), BAX, and cleaved CASP3 genes, which are related to the apoptotic process and are targets of miR-367. In fact, following the downregulation of miR-367 expression, treatment of OS cells with DOX results in apoptosis[181].

Based on this evidence, Wei *et al.* investigated the role of autophagy in OS cells. They observed that treatment with DOX and cisplatin increased the levels of miR-140-5p in OS cells, which stimulates autophagy[182]. Therefore, upregulation of miR-140-5p inhibits cell survival and resistance to DOX and cisplatin, thereby inducing autophagy[182]. The other miRNA involved in the process of chemoresistance is miR-184, which was investigated in OS cell lines by Lin *et al.*[183]. They observed that treatment with DOX induces the time-dependent expression of miR-184 in OS cell lines. It was also observed that miR-184 reduces the number of apoptotic cells after treatment by targeting and inhibiting the BCL2-like 1 (*BCL2L1*) gene, which is involved in the apoptotic process. Therefore, the upregulation of miR-184 and suppression of *BCL2L1* (which inhibits apoptosis) increased the resistance of OS cells to DOX. This study also indicated that downregulation of the expression of miR-184 increased DOX-induced apoptosis[183].

Various studies have aimed to elucidate the molecular mechanisms which confer resistance to treatments, identify miRNAs that could be biomarkers of resistance to DOX and cisplatin, and recognize potential targets for future therapies based on increased sensitivity to chemotherapy. The results indicate that miRNAs are involved in the sensitivity of OS cells to several therapeutic agents [Table 2].

Non-coding miRNA in OS resistance: circRNA and lncRNA

Within the classification of non-coding RNAs are circular RNAs (circRNA) and long non-coding RNAs (lncRNA). circRNAs are characterized by a class of non-coding RNAs with a closed covalently loop without 5'-3' polarity, with a ring structure that gives them resistance and stability to degradation by exonucleases[202]. circRNAs are determined for transcription[203]. They can be classified according to their site of origin and splicing as intron (cell nucleus)[204], exon (cytoplasm)[203], or exon-intron combination[205]. Currently, it has been revealed that circRNA can regulate gene expression at the transcriptional and post-transcriptional level by harvesting RNA-binding proteins, acting as miRNA sponges and nuclear transcriptional regulators[206,207,208]. In OS, circRNA has been implicated in different processes such as proliferation, invasion, apoptosis, and chemoresistance. Zhang *et al.* reported that circ_1569 is upregulated in osteosarcoma[209]. Overexpression of circ_001569 in OS correlated with distant metastasis, advanced tumor stage, and poor prognosis. In U2OS and MG63 cells, circ_001569 expression was elevated and increased cell proliferation. The deletion of circ_001569 decreased the proliferative capacity of OS cells. In addition, the up-regulation of circ_ 001569 promoted resistance to cisplatin, DOX, and MTX, allowing increased cell proliferation and colony formation mediated by Wnt-β-catenin pathway activation. circ_001569 deletion in OS cells decreased the expression of p-GSK3β and β-catenin. The inhibition of Wnt/β-catenin with XAV939 inhibitor decreased resistance to cisplatin, DOX, and MTX; on the contrary, LiCl (Wnt/β-catenin agonist) increased resistance to chemotherapy[209].

Previous studies have identified that overexpression of circPVT1 increases the expression of the ABCB1 gene, related to classical multidrug resistance in OS cells[210]. circPVT1 is derived from a long non-coding RNA region located on chromosome 8q24 within the oncogene PVT1, a cancer susceptibility locus[211]. Kun-

Table 2. miRNAs regulating mechanisms of drug resistance, autophagy, cancer stem cells, and signaling pathways

miRNA	Alteration	Target gene	Mechanism	Effect on resistance	Drug	Reference
miR-124	Downregulation	*ATMIN PARP1*	DNA damage response	Increase	CPT, VP 16, and DOX	[173]
miR-15b	Downregulation	*WEE1*			DOX	[184]
miR 101	Downregulation	*ATG4,5*	Blockage of autophagy	Increase	DOX	[185]
miR 22	Downregulation	*HMGB1*		Increase	DOX and cisplatin	[186,187]
miR 30a	Downregulation	*BECN1*		Increase	DOX	[188]
miR 199a 5p	Downregulation	*BECN1*		Increase	Cisplatin	[189]
miR 155	Upregulation	*ATG5*	Induction of autophagy	Increase	DOX and cisplatin	[190]
miR 140 5p	Upregulation	*IP3K2*		Increase	DOX and cisplatin	[182]
miR 143	Downregulation	*ATG2B BCL2 LC3-II*	Activation of autophagy and stem cells	Increase	DOX	[191]
miR let 7d	Downregulation or Upregulation	*HMGA2 Lin28B Nanog Oct3,4 Sox2*	Induction of EMT and plastic transition of CSC	Increase	DOX, cisplatin, VP-16, paclitaxel	[192]
miR 29b 1	Downregulation	*CD133 N-Myc Nanog Oct3,4 Sox2*	Reduction of CSC	Increase	DOX, cisplatin, and VP 16	[193]
miR 34c	Downregulation	*NOTCH1 LEF1*	Inhibition of metastasis	Increase	DOX, cisplatin, and MTX	[194]
miR 34b	Downregulation	*PAK1 MDR1*	Induction of cell apoptosis	Increase	DOX, GEM, and MTX	[195]
miR 497	Downregulation	*VEGFA*	Inhibition of proliferation	Increase	Cisplatin	[196]
miR 221	Upregulation	*PTEN*	Promotion of proliferation and inhibition of apoptosis	Increase	Cisplatin	[197]
miR 146b 5p	Upregulation	*ZNRF3*	Induction of migration and metastasis	Increase	DOX, cisplatin, and MTX	[198]
miR-488	Upregulation	*BIM*	Promotion of proliferation, reduction of apoptosis	Increase	DOX	[199]
miR-765	Downregulation	*APE-1*	Inhibition of DNA damage response	Decrease	Cisplatin	[179]
miR-21	Upregulation	*Spry1, Spry2 PTEN*	Inhibition of migration/proliferation	Decrease	Cisplatin	[200] [201]
miR-138	Downregulation	*EZH2*	Inhibition of migration/proliferation	Decrease	Cisplatin	[180]
miR-140-5p	Downregulation	*IP3K2*	Induction of cell apoptosis	Decrease	DOX and cisplatin	[182]
miR-184	Upregulation	*BCL2L1*	Inhibition of cell apoptosis	Decrease	DOX	[183]
miR-367	Upregulation	*BAX, cleaved CASP3, KLF4*	Promotion of metastasis and EMT	Decrease	DOX	[181]

APE-1: Apurinic endonuclease; ATG2B: autophagy-related 2B protein; ATMIN: ataxia telangiectasia mutated interactor; BAX: BCL2 associated X; BCL2: B-cell lymphoma 2 protein; BCL2L1: BCL2-like 1; BECN1: beclin 1; BIM: B-cell lymphoma-like protein 11; CASP3: caspase 3; CPT: camptothecin; CD133: prominin-1; CSC: cancer stem cells; DOX: doxorubicin; EMT: epithelial-to-mesenchymal transition; EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit; GEM: gemcitabine; HMGA2: high mobility group AT-hook 2; HMGB1: high mobility group box 1; IP3K2: inositol 1,4,5 trisphosphate kinase 2; KLF4: Kruppel-like factor 4; LC3-II: light chain 3 type II protein; LEF1: lymphoid enhancer binding factor 1; LIN28B: lin-28 homolog B; *MDR1*: multidrug resistance 1; MTX: methotrexate; Nanog: Nanog homeobox; N-myc: mycn proto-oncogene; NOTCH1: Notch receptor 1; Oct 3 and 4: octamer-binding transcription factor 3 and 4; PAK1: p21 activated protein kinase 1; PARP1: poly(ADP ribose) polymerase 1; PTEN: phosphatase and tensin homolog; RAB10: Ras-related protein 10; Sox2: SRY-box transcription factor 2; Spry1 and -2: sprouty; VEGFA: vascular endothelial growth factor A; VP 16: etoposide; ZNRF3: zinc and ring finger 3.

Peng *et al.* demonstrated that circPVT1 overexpression in OS patients correlated with the presence of

metastasis and shorter survival[210]. The expression of circPVT1 is related to DOX and cisplatin resistance. The downregulation of circPVT1 in OS cells decreased resistance to DOX and cisplatin in OS; it appears that circPVTQ deletion decreases resistance to DOX and cisplatin through downregulation of ABCB1 gene expression[210].

lncRNAs, distinguished by having more than 200 nucleotides, do not encode proteins or their coding is limited[212]. lncRNAs act as competitive endogenous RNA (ceRNA) regulators of miRNA expression and are driven to downstream genes[213]. In addition, similar to circRNAs, their functions depend on their location[214]. lncRNAs are involved in chromatin restoration, epigenetic organization, RNA splicing and phase splicing[215]. lncRNA function is involved in transcriptional and post-transcriptional signaling at the cytoplasmic level[216,217]. Thus, emerging evidence shows that lncRNAs are involved in OS chemoresistance. The lncRNA HOXA transcription at the distal tip (HOTTIP) located at the 5' end of the HOXA cluster potentiates the trimethylation of lysine 4 of histone H3 for the activation of multiple 5'Hoxa genes through the WDR5/MLL complex, which will trigger tumor progression[218,219,220]. Recently, in osteosarcoma, upregulated HOTTIP expression correlates with advanced clinical stage, distant metastasis, and unfavorable prognosis[221]. Li *et al.* demonstrated that upregulated HOTTIP in OS increased the expression of cyclin D1, CDK4, and β-catenin involved in cell cycle progression[222]. Increased HOTTIP expression in MG63 cells promoted S-phase cell cycle and increased cisplatin resistance, which can be reversed by XAV939 (Wnt/β-catenin inhibitor). Other lncRNAs have been implicated in OS proliferation, migration, and risk of metastasis and inhibition of apoptosis, a mechanism implicated in chemotherapy resistance, suggesting an important role of lncRNAs in OS tumorigenesis and chemotherapy resistance[222]. A list of the most relevant circRNAs in drug resistance is presented in Table 3 and IncRNAs in Table 4.

AUTOPHAGY

Autophagy is the process through which cells protect and recycle cellular components (e.g., organelles and damaged proteins) that are degraded by autophagosomes. This process allows cells to survive under stress conditions[18]. Autophagy is one of the most important strategies for malignant tumors to promote survival and induce resistance to chemotherapy[236]. Some miRNAs regulate cytotoxic activity; therefore, these miRNAs can improve the sensitivity of OS drug-resistant cell lines to chemotherapy. Li *et al.* developed a model of OS resistant MG-63 cells[189]. Following treatment with cisplatin, the expression of miRNA-199a-5p was decreased, the regulatory target gene Beclin 1 (*BECN1*) negative, and the expression and proportion of LC3-II to LC3-I were increased. These findings indicate the activation of autophagy. Forced overexpression of miRNA-199a-5p in OS cells resulted in the opposite result, inhibiting autophagy, enhancing the cytotoxicity of cisplatin, and reversing the resistance to cisplatin[189]. Chang *et al.* found that miRNA-101 can block autophagy in OS and improve the chemosensitivity of cells to DOX *in vitro*[185]. The results also reveal that autophagy could be induced with a certain dose of DOX in U2OS cells, while the expression of acidic vesicular organelles and another autophagy-related protein, A2g4, was decreased after transfection with miRNA-101, thereby blocking autophagy[185]; this blockage increased the sensitivity of OS cells to DOX. In autophagy, miRNA-199a-3p can promote multidrug resistance by inhibiting the expression of the target gene adenylate kinase 4 (*AK4*). In this process, the reduction of miRNA-199a-3p can upregulate the activated NF-κB pathway[237]. Regarding autophagy, Wei *et al.* found that miRNA-140-5p was upregulated in OS cells treated with cisplatin and DOX[182]. This effect inhibited the inositol 1,4,5-trisphosphate kinase 2 (*IP3K2*) gene and increased autophagy, leading to the development of drug resistance in OS[182]. The use of chloroquine for reversing the autophagy mechanism is currently being explored in various types of tumors. In OS, chloroquine blocks the autophagic process in cisplatin-resistant OS cells; combination with rapamycin enhances the antitumor effect of this agent[238,239]. Autophagy has been shown to be involved in the maintenance of OS cancer stem cell (CSC) characteristics[240]. Stemness in CSCs is

Table 3. List of circular RNAs involved in drug resistance in osteosarcoma

circRNA	Alteration	Target gene	Mechanism	Effect on resistance	Drug	Reference
circ_001569	Upregulation	*Wnt/β catenin*	Promotes Proliferation	Increase	Cisplatin, DOX and MTX	[209]
circPVT1	Upregulation	*ABCB1*	Promotes proliferation	Increase	DOX and cisplatin	[210]
		miR-137/TRIAP1	Reduce apoptosis			[223]
circ_0004674	Upregulation	*circ 0004674/miR-490-3p/ABCC2*	Promotion of proliferation, migration, cell cycle progression, and reduction of apoptosis	Increase	DOX	[224]
		circ 0004674/miR-1254/EGFR				[225]
		circ_0004674/miR-142-5p/MCL-1 miR-342-3p/FBN1 Wnt/β -catenina				[226]
circ_0081001	Upregulation	*miR-494-3p/ TGM2*	Promotion of proliferation, metastasis, and reduction of apoptosis	Increase	MTX	[227]
circ_0000073	Upregulation	*miR-145-5p/NRAS miR-151-3p/NRAS*	Promotion of proliferation, migration (invasion), metastasis, and reduction of apoptosis	Increase	MTX	[228]
circPRDM2	Upregulation	*miR-760/EZH2*	Promotion of proliferation, migration (invasion), and reduction of apoptosis	Increase	DOX	[229]
circ-CHI3L1.2	Upregulation	*miR-340-5p/LPAATβ*	Inhibit EMT, migration (invasion) and reduction of apoptosis	Increase	Cisplatin	[230]

ABCB1: ATP-binding cassette subfamily B member 1; ABCC2: ATP binding cassette subfamily C member 2; DOX: doxorubicin; EGFR: epidermal growth factor receptor; EMT: epithelial-to-mesenchymal transition; EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit; FBN1: fibrillin-1; TGM2: transglutaminase-2; LPAATβ: lysophosphatidic acid acyltransferase β; MCL-1: myeloid cell leukemia-1; MTX: methotrexate; NRAS: NRAS proto-oncogene GTPase; TRIAP1: TP53 regulated inhibitor of apoptosis 1.

Table 4. List of long non-coding RNAs involved in drug resistance in osteosarcoma

lncRNA	Alteration	Target gene	Mechanism	Effect on resistance	Drug	Reference
HOTTIP	upregulated	*Wnt/β- catenin*	Promotion of proliferation and cell cycle progression	Increase	Cisplatin	[222]
ENST00000563280 (FOXC2-AS1)	upregulated	*ABCB1 HIF1A FOXC2*	Induction of migration and metastasis	Increase	DOX	[37,231,232]
LUCAT1	upregulated	*miR-200c/ABCB1*	Promotion of proliferation and migration (invasion)	Increase	MTX	[37,233]
NR-036444 (FENDRR)	Downregulation	*ABCB1 HIF-1α FOXC2*	Induction of migration and metastasis	Increase	DOX	[37,231]
		ABCB1/ABCC1	Promotion of apoptosis			[234]
LINC00161	Downregulation	*miR-645/IFIT2*	Promotion of apoptosis	Decrease	Cisplatin	[235]

ABCB1: ATP-binding cassette subfamily B member 1; ABCC1: ATP binding cassette subfamily C member 1; DOX: doxorubicin; MTX: methotrexate; FENDRR: FOXF1 adjacent non-coding developmental regulatory RNA; FOXC2-AS1: Forkhead box C2 antisense RNA 1; HIF-1α: hypoxia-inducible factor 1 alpha; IFIT2: interferon-induced protein with tetratricopeptide repeats 2; LINC00161: long intergenic non-protein coding RNA 161; LUCAT1: lung cancer-related transcript 1.

favored by autophagy in some types of tumors, e.g., breast cancer, pancreatic ductal adenocarcinoma, colon cancer, *etc*. Osteosarcoma CD271+ cells have stem cell characteristics that seem dependent on autophagic activity, with an increased expression of essential autophagy genes such as *Beclin1*, *LC3B*, *Atg5*, and *Atg7* compared to CD271 OS cells. Autophagy confers to CD271+ OS cells several advantages including resistance to hypoxic conditions, chemotherapy resistance to cisplatin and epirubicin, and tumorigenicity

compared to CD271- OS cells. Autophagy-deficient CD271+ OS cells had no remarkable difference with autophagy-deficient CD271-OS, suggesting an important contribution of autophagy to the stemness of CD271+ OS cells under stress conditions. Interestingly, the inhibition of the autophagy in CD271+ OS cells reversed chemotherapy resistance, resulting in a potential pathway to be explored in OS chemotherapy resistance[241].

CANCER STEM CELLS

Cancer stem cells are a subset of cells within the microenvironment that self-renew and aberrantly mature into OS cells. These cells are characterized by a highly active DNA repair mechanism or enhanced protection against reactive oxygen species (ROS), increased expression of markers such as CD117 and CD133, and upregulation of *MDR1* protein transport genes (e.g., ABCG2) related to resistance to chemotherapy with DOX or drug efflux pumps[18,242], which correspond to a combination of intrinsic and extrinsic factors contributing to CSC-mediated resistance to chemotherapy in OS. CSCs have an effective autophagy system and a complex EMT regulator capacity that lead to adaptation to TME stress conditions such as nutritional, metabolic, and oxygen privation[243]. Other potential biomarkers have been reported in CSCs. Honoki *et al.* found an association of high expression of ALDH-1 (aldehyde dehydrogenase 1) with resistance to chemotherapy, as well as its metastatic potential[244]. Schiavone *et al.* mentioned at least 20 biomarkers expressed by stem cells in OS, some of them related to chemoresistance, such as Oct4, Nanog, Sox2, CD24, CD44, Stro-1, CD133, KLF4, CBX3, and ABCA5[245]. Despite recognizing the role of stem cells in this tumor, an important limitation to making them a potential therapeutic target for treatment or research is that the tumor stem cell population in OS that corresponds to a population of < 1%[246]. Other cells with pluripotent stem cell characteristics are MSCs, which are cells highly associated with carcinogenesis, disease progression, metastasis, and drug resistance. Funes *et al.* evidenced MSC transformation in OS related to genetic alterations, such as p53 (*TP53*) and *RB* gene deficiency[247]. For example, Rb pathway deficiency is sufficient to induce tumor growth and progression in hypoxic conditions and favor immune system infiltrations; both conditions may contribute to chemotherapy resistance. Mutations of p53 and Rb occur in one clone of OS cells and drive to chromosomal instability and further pro-tumoral events inherited to next-generation clones, resistant to chemotherapy[33,248]. Additionally, aneuploidization and genomic loss of p16/CDKN2A are common causes of the transition from MSC to OS cells, and loss of p16/CDKN2A protein is a predictor of poor response to chemotherapy and worse overall survival in OS patients[84,249,250]. Tu *et al.* observed that activation of the signal transducer and activator of transcription 3 (STAT3) by IL-6 regulated MSCs and induced resistance to DOX and cisplatin[251]. Furthermore, the expression of p-STAT3 contributed to high resistance to chemotherapy in clinical samples of OS cells[251]. Stem cells are involved in OS tumorigenesis and treatment response and are an important research topic in this tumor.

CONCLUSIONS

Although the application of chemotherapeutic agents contributes greatly to the effective treatment of OS, the emergence of acquired multidrug resistance remains a serious challenge. This is particularly important when using drugs that have shown greater efficacy in these tumors (e.g., cisplatin, DOX, and MTX). Several universal mechanisms underlying acquired resistance have been discovered, including drug transport, drug metabolism, aberrant drug targets, DNA damage response, apoptosis evasion, autophagy, epithelial-to-mesenchymal transition, *etc*. These mechanisms offer new directions for future management strategies that could improve oncological outcomes in patients with OS. Anti-angiogenic TKIs show that targeted therapy can improve the prognosis of this type of cancer. Novel pan-HER pathway TKIs, such as afatinib, may be effective in treating OS. New therapies that target tumor ECM pathways and the cell cycle may be helpful in overcoming resistance to chemotherapy and targeted therapy in OS.

DECLARATIONS

Authors' contributions

Conceptualization, visualization, and research: Garcia-Ortega DY, Cabrera-Nieto SA, Caro-Sánchez HS, Cruz-Ramos M
Supervision: Garcia-Ortega DY, Cabrera-Nieto SA, Caro-Sánchez HS, Cruz-Ramos M
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All authors declared that there are no conflicts of interest.

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Consent for publication

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Cancer Drug Resistance

Review

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Insight into the molecular mechanisms of gastric cancer stem cell in drug resistance of gastric cancer

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Abstract

Gastric cancer (GC) is one of the most common causes of cancer-related death worldwide, and gastric cancer stem cells (GCSCs) are considered as the major factor for resistance to conventional radio- and chemotherapy. Accumulating evidence in recent years implies that GCSCs regulate the drug resistance in GC through multiple mechanisms, including dormancy, drug trafficking, drug metabolism and targeting, apoptosis, DNA damage, epithelial-mesenchymal transition, and tumor microenvironment. In this review, we summarize current advancements regarding the relationship between GCSCs and drug resistance and evaluate the molecular bases of GCSCs in drug resistance.

Keywords: Gastric cancer, gastric cancer stem cells, drug resistance, chemotherapy, molecular mechanisms

INTRODUCTION

According to GLOBOCAN estimates in 2020, gastric cancer (GC) is the fifth cause of global cancer incidence and the fourth leading cause of cancer mortality[1]. The incidence rates of GC vary widely across the world, with the highest rates in East Asia and Eastern Europe[1]. GC, most cases of which are gastric adenocarcinoma (GAC), is histologically divided into two subtypes [intestinal and diffuse (Lauren

classification)][2] or four subtypes [papillary, tubular, mucinous, and poorly cohesive (WHO classification)][3]. Based on genomic and epigenomic alterations, the most well-defined molecular-based classification systems include The Cancer Genome Atlas (TCGA) classification [EBV positive (EBV), microsatellite instable (MSI), genomically stable (GS), and chromosomal instable (CIN)][4] and the Asian Cancer Research Group (ACRG) classification [microsatellite instable (MSI), microsatellite stable TP53 inactive (MSS/TP53 inactive), MSS TP53 active (MSS/TP53 active), and MSS with epithelial–mesenchymal transition (EMT) features (MSS/EMT)][5]. Despite advances in the field of early diagnosis in GC, most cases are still diagnosed at an advanced stage[6] with unresectable or metastatic disease. Although current systemic treatments, including surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy [Table 1] for advanced GC patients, have been considerably improved during recent decades, most patients with advanced GC die from tumor relapse and metastasis. The prognosis of advanced and metastatic GC remains poor, and the 5-year survival rate is < 10%[7].

Early GC patients can be cured with surgery alone. For advanced unresectable patients, chemotherapy represents the backbone of systemic therapy, and chemotherapy with or without radiotherapy has been integrated into standard-of-care therapies. Cytotoxic chemotherapy has been demonstrated to be effective for advanced GC patients, and the common cytotoxic chemotherapy drugs include fluoropyrimidines (e.g., fluorouracil, capecitabine, and S-1), platinums (e.g., cisplatin and oxaliplatin), taxanes (e.g., paclitaxel and docetaxel), topoisomerase inhibitors (e.g., irinotecan), and anthracyclines (e.g., doxorubicin and epirubicin). In patients with human epidermal growth factor 2 (HER2)-negative, advanced gastric adenocarcinoma, the current first-line treatment consists of two- or three-drug regimens. Doublet therapies are the combination of platinum derivatives (cisplatin and oxaliplatin) and fluoropyrimidine analogs (5-fluorouracil, capecitabine, and S-1). Three-drug regimens are the triplet combinations adding taxanes or anthracyclines (doxorubicin and epirubicin) to the doublet regimen. Table 2 summarizes the landmark trials for first-line treatment of advanced gastric cancer. However, the clinical benefit from these treatments is limited due to the toxicity of chemotherapeutic drugs and the development of drug resistance.

More recently, targeted therapies have been developed for gastric cancer patients. These include trastuzumab, lapatinib, and margetuximab for epidermal growth factor receptor-2 (HER-2); bevacizumab for vascular endothelial growth factor (VEGF); ramucirumab, apatinib, and regorafenib for vascular endothelial growth factor receptor (VEGFR); cetuximab and panitumumab for endothelial growth factor receptor (EGFR); bemarituzumab for fibroblast growth factor receptor (FGFR); everolimus for mTOR; and zolbetuximab for Claudin 18.2. Various targeted therapy approaches have been investigated; however, several clinical trials in gastric cancer have failed due to the tumor heterogeneity and the difficulty of screening the beneficiary population for targeted therapeutic drugs. Furthermore, immunotherapy is being developed to block the binding of ligands to checkpoint receptors and re-activate the human cellular immune response. These are immune checkpoint inhibitors (ICIs) including nivolumab and pembrolizumab as PD-1 inhibitors, durvalumab and avelumab as PD-L1 inhibitors, and ipilimumab and tremelimumab as CTLA-4 inhibitors. Several trials have demonstrated that the benefits of immunotherapy only or with cytotoxic chemotherapy are relatively limited[17]. Thus, with relatively low response rates, the use of immunotherapy has only led to limited approval in the second-line treatment setting for GC[18]. More and more promising targeted therapies and immunotherapies are being investigated, and these will likely further improve outcomes for patients. Table 3 summarizes the landmark trials for targeted therapy and immunotherapy of advanced gastric cancer.

Drug resistance leads to pharmacological treatment failure and poor outcomes for advanced GC patients. The mechanisms of drug resistance of GC are divided into seven groups, according to the previously

Table 1. A summary of systemic treatments in gastric cancer

Treatment approaches	Mechanisms	Regimen	Efficacy	Adverse effects
Surgery	Surgical resection	Open surgery, laparoscopic surgery, endoscopic resection, robotic surgery	Primary choice for early-stage GC	Low
Chemotherapy	Target and kill fast-dividing cells	Fluoropyrimidines, platinums, taxanes, irinotecan, *etc.*	The standard-of-care treatment for advanced GC	Damage to normal and healthy tissues
Radiotherapy	Ionizing radiation to target and kill tumor tissue	Ionizing radiation	Curative and palliative treatment	Damage to normal and healthy tissues
Targeted therapy	Target the specific molecules (HER2, VEGF, VEGFR, *etc.*)	Monoclonal antibodies and small molecule inhibitors	Use in combination with chemotherapy in first- and second-line settings	Relatively low
Immunotherapy	Block the binding of ligands to checkpoint receptors and re-activate the human cellular immune response	Immune checkpoint inhibitors (PD-1, PD-L1, and CTLA-4)	Use in second- and third-line settings	Relatively low

GC: Gastric cancer; HER2: human epidermal growth factor receptor 2; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; PD-1: programmed cell death protein 1; PD-L1: programmed cell death 1 ligand 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4.

Table 2. Landmark trials in first-line treatment of advanced gastric cancer

Studies	Treatment regimen	ORR	mPFS (mo), *P*-value	mOS (mo), *P*-value	Reference
MacDonald, *et al.* 1980	Fluorouracil, doxorubicin, mitomycin (FAM)	42%	NR	5.5	[8]
Wils, *et al.* 1991	Fluorouracil , doxorubicin, methotrexate (FAMTX) *vs.* FAM	41%/9%	NR	9.7/6.7	[9]
Webb, *et al.* 1997	Epirubicin, cisplatin, fluorouracil (ECF) *vs.* FAMTX	45%/21%	7.4/3.4, P = 0.00006	8.9/5.7, P = 0.0009	[10]
Van Cutsem, *et al.* 2006	Cisplatin, fluorouracil (CF) *vs.* Docetaxel,cisplatin, fluorouracil (DCF)	37%/25%	5.6/3.7, P < 0.001	8.2/9.6, P = 0.02	[11]
Cunningham, *et al.* 2008	Epirubicin, cisplatin, fluorouracil (ECF) *vs.* Epirubicin, cisplatin, capecitabine (ECX) *vs.* Epirubicin, oxaliplatin, fluorouracil (EOF) *vs.* Epirubicin, oxaliplatin, capecitabine (EOX)	41%/46%/42%/48%	6.2/6.7/6.5/7.0	9.9/9.9/9.3/11.2	[12]
Kang, *et al.* 2009	Cisplatin, capecitabine (XP) *vs.* Cisplatin, fluorouracil (FP)	41%/29%	5.6/5.0, P < 0.001	10.5/9.3, P = 0.008	[13]
Shah, *et al.* 2010	DCF, granulocyte stimulating factor (G-CSF) *vs.* modified DCF (mDCF)	33%/49%	6.5/9.7, P = 0.2	12.6/18.8, P = 0.007	[14]
Koizumi, *et al.* 2014	S-1, Docetaxel *vs.* S-1	38.8%/26.8%	5.3/4.2, P < 0.001	12.5/10.8, P = 0.032	[15]
Guimbaud, *et al.* 2014	epirubicin, cisplatin, capecitabine (ECX) *vs.* Folinic acid, Fluorouracil, Irinotecan (FOLFIRI)	39.2%/37.8%	5.3/5.8, P = 0.96	9.5/9.7, P = 0.95	[16]

ORR: Objective response rates; mPFS (mo): median progression-free survival (months); mOS (mo): median overall survival (months); NR: not reported.

proposed classification[40]: change in drug intracellular concentration (MOC-1), change in drug metabolism (MOC-2), change in drug targets (MOC-3), change in DNA repair (MOC-4), change in apoptosis and survival (MOC-5), change in tumor cell microenvironment (MOC-6), and phenotypic transformation (MOC-7) [Figure 1]. We summarize the updated knowledge of the molecular mechanisms attributed to drug resistance in GC in Figure 2.

Table 3. Landmark trials in targeted therapy and immunotherapy [immune checkpoint inhibitors (ICIs)] for the treatment of advanced GC

Items	Drugs	Treatment regimen	ORR, *P*-value	mPFS (mo), *P*-value	mOS (mo), *P*-value	Reference
Targeted therapies	**HER-2**					
	Trastuzumab	Capecitabine or 5- FU plus cisplatin with Trastuzumab *vs.* Capecitabine or 5- FU plus cisplatin	47%/35%, *P* = 0.0017	6.7/5.5, *P* = 0.0002	13.8/11.1, *P* = 0.0046	[19]
	Trastuzumab	TrastuzumabDeruxtecan *vs.* Irinotecan or Paclitaxel	51%/14%, *P* < 0.001	5.6/3.5, *P* = 0.01	12.5/8.4	[20]
	Lapatinib	Paclitaxel with Lapatinib *vs.* Paclitaxel	27%/9%, *P* < 0.001	5.4/4.4, NS	11.0/8.9, NS	[21]
	Lapatinib	Capecitabine plus oxaliplatin with Lapatinib *vs.* Capecitabine plus oxaliplatin	53%/39%, *P* = 0.0031	6.0/5.4, *P* = 0.0381	12.2/10.5, NS	[22]
	Margetuximab	Margetuximab *vs.* Trastuzumab	25%/14%, *P* < 0.001	5.8/4.9, *P* = 0.03	21.6/19.8, NS	[23]
	VEGF					
	Bevacizumab	Capecitabine plus cisplatin with Bevacizumab *vs.* Capecitabine plus cisplatin	46%/37.4%, *P* = 0.0315	6.7/5.3, *P* = 0.0037	12.1/10.1, NS	[24]
	VEGFR					
	Ramucirumab	Ramucirumab *vs.* placebo	3%/3%, NS	2.1/1.3, *P* < 0.0001	5.3/3.8, *P* = 0.047	[25]
	Ramucirumab	Paclitaxel with Ramucirumab *vs.* Paclitaxel	28%/16%, *P* = 0.0001	4.4/2.9, *P* = 0.0001	9.6/7.4, *P* = 0.017	[26]
	Apatinib	Apatinib *vs.* placebo	2.84%/0%, NS	2.6/1.8, *P* < 0.001	6.5/4.7, *P* = 0.0149	[27]
	Regorafenib	Regorafenib *vs.* placebo	NR	2.6/0.9, *P* < 0.001	5.8/4.5, NS	[28]
	EGFR					
	Cetuximab	Capecitabine plus cisplatin with Cetuximab *vs.* Capecitabine plus cisplatin	30%/29%, NS	4.4/5.6, NS	9.4/10.7, NS	[29]
	Panitumumab	Epirubicin, oxaliplatin, and capecitabine with Panitumumab *vs.* Epirubicin, oxaliplatin, and capecitabine	46%/42%, NS	6.0/7.4, NS	8.8/11.3, *P* = 0.013	[30]
	FGFR					
	Bemarituzumab	Modified FOLFOX6 with Bemarituzumab *vs.* modified FOLFOX6	47%/33%	9.5/7.4, *P* = 0.073	Not reached/12.9, *P* = 0.027	[31]
	mTOR					
	Everolimus	Everolimus *vs.* placebo	4.5%/2.1%	1.7/1.4, *P* < 0.001	5.4/4.3, NS	[32]
	Claudin 18.2					
	Zolbetuximab	EOX with Zolbetuximab *vs.* EOX	39.0%/25.0%, *P* = 0.034	7.5/5.3, *P* < 0.0005	13.0/8.3, *P* < 0.0005	[33]
Immunotherapy-Immune Checkpoint Inhibitors (ICIs)	**PD-1**					
	Nivolumab	Nivolumab *vs.* placebo	11.2%/0%, *P* = 0.0088	6.1/1.61, *P* < 0.0001	11.6/5.26, *P* < 0.0001	[34]
	Nivolumab	CTx (S-1 or capecitabine plus oxaliplatin) with Nivolumab *vs.* CTx (S-1 or capecitabine plus oxaliplatin)	57.5%/47.8%, *P* = 0.0088	10.45/8.34, *P* = 0.0007	17.45/17.15, NS	[35]
	Pembrolizumab	Pembrolizumab *vs.* paclitaxel	16%/14%	1.5/4.1, *P* = 0.0007	9.1/8.3, *P* = 0.0421	[36]
	PD-L1					

Durvalumab	Durvalumab plus tremelimumab, 2 L *vs.* Durvalumab, 2 L *vs.* Tremelimumab, 2 L *vs.* Durvalumab, tremelimumab, 3 L *vs.* Durvalumab, tremelimumab, 2 L/3 L	7.4%/0%/8.3%/4.0%	1.8/1.6/1.7/1.8	3.4/3.2/7.7/10.6,	[37]
Avelumab	Avelumab *vs.* chemotherapy (paclitaxel or irinotecan)	2.2%/4.3%	1.4/2.7, NS	4.6/5.0, NS	[38]
CTLA-4					
Ipilimumab	Ipilimumab *vs.* first-line chemotherapy	1.8%/7.0%	2.72/4.90, *P* = 0.034	12.7/12.1	[39]
Tremelimumab	Durvalumab plus tremelimumab, 2 L *vs.* Durvalumab, 2 L *vs.* Tremelimumab, 2 L & Durvalumab, tremelimumab, 3 L *vs.* Durvalumab, tremelimumab, 2 L/3 L	7.4%/0%/8.3%/4.0%	1.8/1.6/1.7/1.8	3.4/3.2/7.7/10.6,	[37]

ORR: Objective response rates; mPFS (mo) : median progression-free survival (months); mOS (mo) : median overall survival (months); HER-2: epidermal growth factor receptor-2; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; EGFR: endothelial growth factor receptor; FGFR: fibroblast growth factor receptor; mTOR: mammalian target of rapamycin; PD-1: programmed cell death protein 1; PD-L1: programmed cell death 1 ligand 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; NS: no significant difference; NR: not reported.

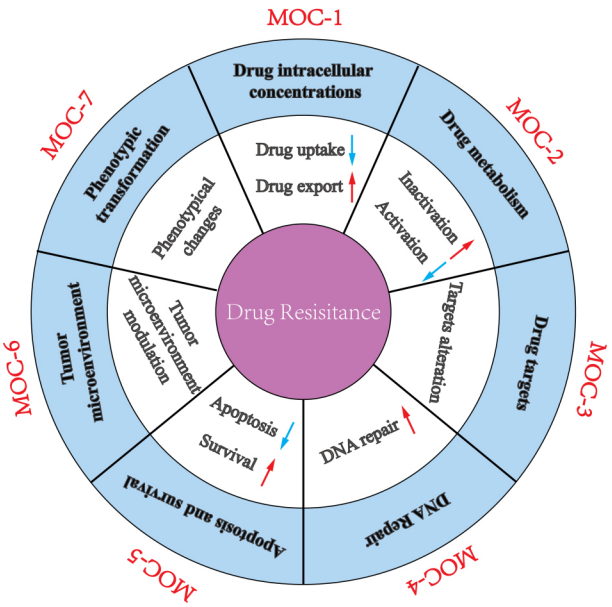

Figure 1. A schematic diagram depicting the molecular mechanisms accounting for drug resistance in gastric cancer.

Cancer stem cells (CSCs) are a subpopulation of stem cell-like cancer cells that are responsible for cancer pathogenesis including initiation, development, drug resistance, metastasis, and cancer recurrence[41-43]. In recent years, accumulating evidence indicates the presence of CSCs in various types of cancers, including brain[44], breast cancer[45], head and neck cancer[46], renal cancer[47], colon cancer [48-50], pancreatic cancer[51-52], liver cancer[53], lung cancer[54], prostate cancer[55], and melanoma[56], and targeting CSCs may be essential to prevent tumor relapse and spread[57]. Moreover, growing evidence suggests that there are several signaling pathways preferentially associated with CSCs[58-60], including Hedgehog, Notch, WNT/β-catenin, JAK/STAT, PI3K/PTEN, and NF-κB pathways, which contribute to the survival, self-renewal, and differentiation

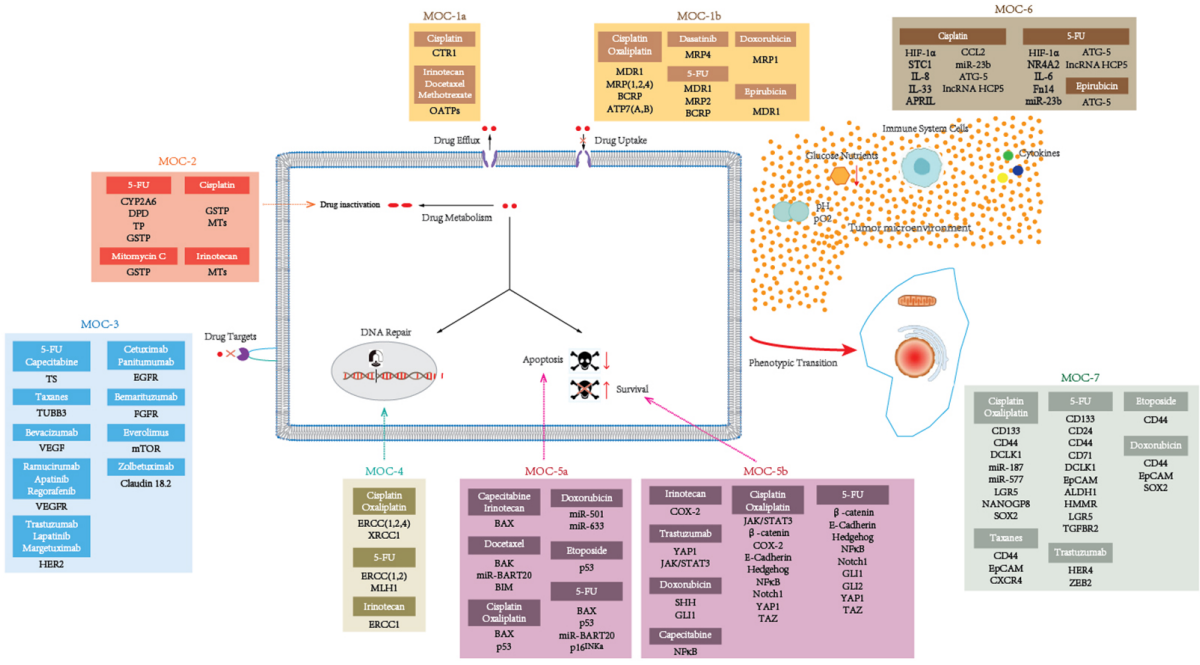

Figure 2. Proteins and non-coding RNAs accounting for drug resistance in gastric cancer. This figure is based on the work of Marin *et al.*[40].

properties of CSCs[61].

CSC-targeting therapies are currently being investigated to reverse chemoresistance, including chemotherapeutic and biological agents that target stemness pathways including Hedgehog, Notch, Hippo/YAP1, JAK/STAT, and Wnt/β-catenin pathways; cancer stem cell surface markers including CD24, CD44, CD54, CD71, CD90, CD133, ALDH, CXCR4, EpCAM, LGR5, Sox2, and Oct4; the CSC microenvironment; and others[62-64]. However, these current strategies to target CSCs are not specific to CSCs, leading to toxic effects on normal tissues.

CSCs in GC were first identified from a panel of human GC cell lines[65]. Cancer stem cells from either human GC cell lines or tumor tissues were isolated using cell surface markers such as CD24, CD44, CD54, CD71, CD90, CD133, Lgr5, ALDH1, EpCAM, and CXCR4[63, 66-67]. Although studies suggest the presence of gastric cancer stem cells (GCSCs), the origin of GCSCs is currently unclear and controversial. Two major hypotheses propose that GCSCs are derived from normal gastric stem cells (GSCs) or from bone marrow-derived mesenchymal stem cells (BM-MSCs)[68, 69].

In recent years, growing evidence shows that GCSCs play important roles in drug resistance in GC. Thus, understanding GCSC functions and their roles in drug resistance, as well as defining the molecular mechanisms of drug resistance, will help identify potential anticancer drug targets and develop new chemotherapeutic drugs and effective therapeutic strategies to improve the clinical outcomes of GC patients. In this review, we summarize our current understanding of the roles of GCSCs in GC drug resistance, as well as provide a comprehensive analysis of the potential molecular mechanisms by which CSCs contribute to drug resistance in GC.

GCSCS AND DRUG RESISTANCE

Substantial studies have demonstrated that GCSCs are resistant to conventional radio-/chemotherapy. Aldehyde dehydrogenase (ALDH) is generally highly expressed in stem cells and considered as a CSC marker[70]. Gastric cancer cells with high expression of ALDH showed strong resistance to 5-fluorouracil (FU) and cisplatin; thus, high expression of ALDH in GC cell lines is believed to play a key role in resistance to chemotherapeutic drugs in GC[71,72]. Similarly, upregulation of LGR5, another GCSC marker, significantly enhanced cell stemness and drug resistance in MGC803 cells[73]. Further studies have shown that LGR5+ GCSCs are resistant to cisplatin treatment[74]. We recently showed that CD44+/CD54+ GCSCs isolated from cancer tissues can survive and expand after treatment with 5-FU and cisplatin[75]. Consistent with our results, another study showed that KHDRBS3 plays an important role in the acquisition of 5-FU resistance by regulating CD44 variant expression[76]. These results show that GCSCs play a key role in the acquisition of drug resistance in GC.

Accumulating evidence suggests the drug resistance capability of GCSCs is significantly higher than that of GC cells, and GCSCs can be enriched in GC after chemotherapy. Compared with GC cells, GCSCs showed stronger resistance to chemotherapeutic drugs 5-FU and oxaliplatin[77]. CSCs can be isolated or enriched by CSC-specific surface markers or through stem cell side population (SP) analysis[78]. Similarly, GCSCs isolated from GC cell lines by the SP method showed more resistance to chemotherapy[79]. Further study showed that CD44+ GCSCs isolated from tumor tissues were significantly enriched after treatment with 5-FU[80]. Another study demonstrated that ALDH+ CSCs in GC cell cultures can be enriched after treatment with cisplatin and 5-fluorouracil[81]. Meanwhile, clinical studies have revealed that resistance to anticancer drugs of GC is mainly associated with GCSCs. Patients with high CD133 expression exhibited stronger drug resistance, higher relapse rate, and lower five-year survival rate compared with patients with low CD133 expression[82]. Similarly, patients with high CD44 and CD133 expression showed worse survival[83]. Furthermore, expression of LGR5 and CD133 was identified to be significantly associated with poor clinical outcomes, and patients who are LGR5+ and CD133+ showed a lower overall survival rate than those who are LGR5- and CD133-[84]. The results from a phase II clinical trial show that GC patients with high expression CD44 who received chemotherapy with vismodegib, a hedgehog inhibitor, held a survival advantage[85]. Therefore, GCSCs are a major factor in GC resistance to radiation and chemotherapy.

THE UNDERLYING MECHANISMS FOR GCSCS REGULATING THE DRUG RESISTANCE

Drug resistance is a multifactorial phenomenon involving various components and multiple interrelated pathways, which work together to contribute to the development of this phenomenon. Various CSC-associated signaling pathways and molecular mechanisms have been described as implicated in CSC drug resistance[86]. To our knowledge, the underlying molecular mechanisms by which GCSCs contribute to chemoresistance include dormancy, drug trafficking, drug metabolism and targeting, apoptosis and cell death, DNA damage, epithelial-mesenchymal transition (EMT), and tumor microenvironment. The molecular mechanisms attributed to drug resistance in GCSCs are described below based on the previously proposed classification (MOC-1-7)[40], and a schematic outline is summarized in Figure 3.

Dormancy

Tumor dormancy contributes to the development of chemoresistance, metastasis, and cancer recurrence. CSCs are frequently in a quiescent state in which CSCs can remain in the G0/G1 stage with a low proliferation rate[87,88]. As most conventional chemotherapeutic drugs target proliferating cells, quiescence properties support CSCs to become resistant to radio- and chemotherapy[72,89-90]. Accordingly, 5-FU-resistant GC cells with CSC features were found to be mainly quiescent cells, which remained in the G0/1 phase[91]. Similarly, IL-17 enhances the proliferative capacity of quiescent gastric stem cells[92], potentially promoting

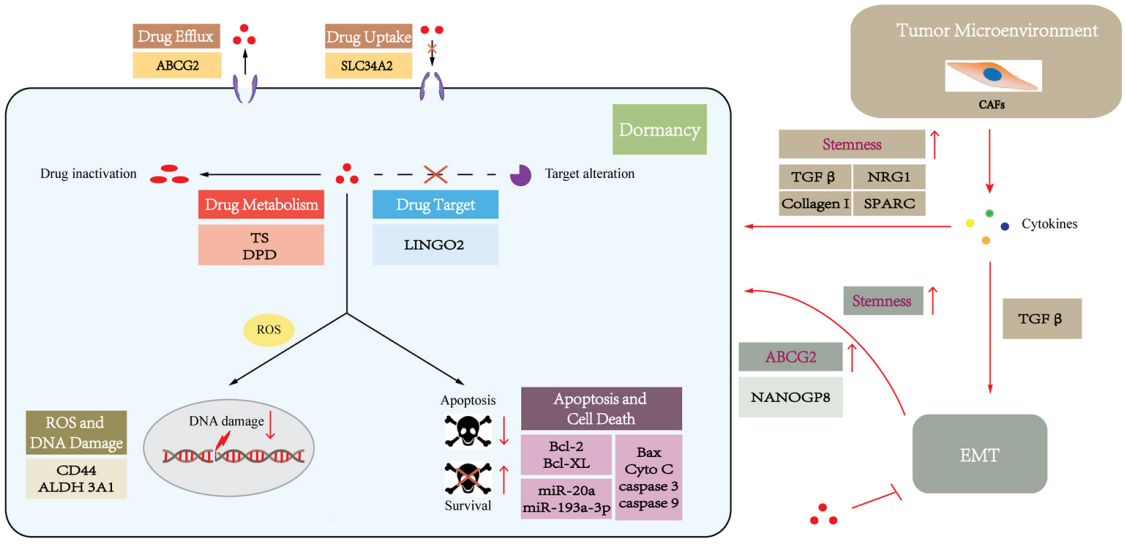

Figure 3. A schematic diagram depicting the molecular mechanisms accounting for chemoresistance in GCSCs. This figure is based on the work of Marin *et al.*[40].

these transformed GCSCs to be sensitive to chemotherapy.

Changes in drug uptake, efflux, metabolism, and targeting

Reduced drug uptake

One of the most studied mechanisms of cancer drug resistance is reducing the uptake of drugs. The uptake of drugs into tumor cells can be active transportation mediated by the membrane transporters. Most of these membrane transporters are solute carrier (SLC) proteins, which play an essential role in drug uptake. The SLC protein family contains more than 300 proteins that mediate the absorption of multiple types of substrates, including amino acids, sugars, organic cations, anions, *etc*., as well as chemotherapeutic drugs[93]. Unexpectedly, SLC34A2 was found to be increased in CD44+ GCSCs, and suppression of SLC34A2 in GCSCs reduced the effects of chemoresistance[94]. This result is consistent with SLC34A2 potentially having an oncogenic role in GC cells[95]. However, the detailed molecular mechanism remains unclear, which requires further investigation.

Increased drug efflux

Another mechanism of drug resistance is associated with the increased efflux of cytotoxic drugs by active ATP-binding cassette (ABC) transporter proteins, which is known as "drug efflux". Forty-eight ABC transporter members have been identified in humans and are divided into seven distinct subfamilies (ABCA-ABCG) with different functions. Only 13 ABC transporters (ABCA2/3, ABCB1/2/5, ABCC1/2/3/4/5/6/10, and ABCG2) have been directly associated with chemoresistance[96]. Three major ABC transporters, including P-glycoprotein (P-gp/MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/ABCG2), have been found in various drug-resistant cancer cell lines and tissues and studied extensively for their correlation to multidrug resistance (MDR)[97]. These ABC transporters can lower the intracellular drug concentration by pumping out chemotherapy-based agents, including vinblastine, vincristine, doxorubicin, daunorubicin, actinomycin-D, and taxanes[98,99].

CSCs express a high number of ABC transporter proteins on their cell surface[64,86], and ABC transporters in CSCs have been shown to play an important role in drug resistance[72,100-101]. Therefore, ABC transporters are widely used as surface markers for CSC identification and isolation[57].

ABCG2 is a major multidrug resistance pump, which is a downstream target of the sonic hedgehog (SHH)-glioma-associated oncogene homolog (GLI) signaling pathway[102]. Recent studies have indicated ABCG2 plays a pivotal role in drug resistance in GCSCs. CD44+/Musashi-1+ GCSCs with increased expression of ABCG2 exhibited resistant to doxorubicin[103]. Moreover, the inactivation of SHH-GLI signaling pathways decreased ABCG2 expression, rendering GCSCs more chemosensitive to doxorubicin[103]. This result implies that ABCG2 is a potential therapeutic target against CSCs to overcome drug resistance. Moreover, inhibition of ABCG2 expression by genistein, which is the predominant isoflavone in soy products, could inhibit gastric cancer stem cell-like features and reduce the chemoresistance of GCSCs[104]. Another study also demonstrated miR-132 could enhance cisplatin resistance in LGR5+ GCSCs via the SIRT1/CREB/ABCG2 signaling pathway[74]. However, the roles of other ABC transporter proteins of GCSCs in drug resistance are not clearly defined, and further investigations are needed to explore the roles of ABCs in cancer therapies against GCSCs.

Altered drug metabolism

Thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) are both key 5-FU metabolic enzymes. TS is a target enzyme of 5-FU, and 5-FU exerts an anticancer effect by its conversion into fluorodeoxyuridine monophosphate (FdUMP) which can form a ternary complex with TS to cause suppression of the de novo synthesis of dTMP. DPD is the initial and rate-limiting enzyme that translates 5-FU into metabolites without cytotoxicity. TS and DPD, which are representative markers of 5-FU resistance, were shown to be significantly upregulated in a 5-FU-resistant CSC-like cell population in GC[76]. Hence, metabolic inactivation or alteration of the anticancer drugs in GCSCs enable CSCs to resist therapy and strengthen their stemness.

Changed drug targeting

Drug targeting altering by changing the expression and function of drug targets is also one of the major causes of drug resistance. Receptors of tyrosine kinase and its downstream signaling pathway play a pivotal role in carcinogenesis and tumor development and constitute the targets for tyrosine kinase inhibitors (TKIs). For instance, the EGFR signaling pathway is involved in the pathogenesis and progression of cancers by activation of either RAS/RAF/MEK/ERK or PI3K/AKT/mTOR cascade[105]. The activation of MEK, a component of the EGFR/Ras/RAF/MEK/ERK signaling pathway, caused drug resistance to MEK inhibitors[106]. Correspondingly, silencing LINGO2, a GCSC-related marker, reduced AKT, ERK, and MEK phosphorylation[107], suggesting that activation of AKT, ERK, and MEK in GCSCs is responsible for chemoresistance to the inhibitors targeting these kinases.

Inhibition of apoptosis and cell death

One of the primary goals of most anticancer agents is to cause tumor-selective cell death. The resistance to apoptosis, one of the key regulatory events leading to cell death, is the hallmark of cancer. Apoptosis occurs through extrinsic and intrinsic pathways that are dependent on caspase activation and mitochondrial outer membrane permeabilization (MOMP), respectively. The extrinsic apoptotic pathway is often related to ligands such as TNF-α, TNF-α-related apoptosis-inducing ligand (TRAIL), and Fas-ligand (FasL) and cell death receptors such as TNFR, TRAILR, FasR, linker proteins, and caspases 3, 6, 7, and 8. The intrinsic pathway is triggered by mitochondrial membrane disturbance following various stimuli including DNA damage and radio-/chemotherapy. Pro-apoptotic proteins such as Bax and Bak, as well as anti-apoptotic

proteins such as Bcl2 and Bcl-XL are involved. Both intrinsic and extrinsic pathways activate caspases and ultimately lead to cell apoptosis. Increasing evidence suggests that disruption of the apoptotic pathway impacts resistance to anticancer drugs in GCSCs. Pro-apoptotic proteins including Bax, cytochrome C, caspase 9a, cleaved caspase 3, and cleaved caspase 9 were observed to be downregulated, while anti-apoptotic proteins Bcl-2 and Bcl-XL were upregulated in CD44+ GC cells compared with CD44- GC cells[108]. Moreover, in CD44+ GC cells, inhibition of miR-193a-3p can induce apoptosis by activating the mitochondrial apoptotic pathway and enhance the chemotherapeutic response of cisplatin[108]. Thus, GCSCs can induce resistance to drug-mediated apoptosis by upregulation or activation of anti-apoptotic proteins or downregulation or mutation of pro-apoptotic proteins. Similarly, miR-20a could increase cisplatin resistance in GC cells via modulating the anti-apoptotic factors livin and survivin[109,110], whereas miRNA-19b, -20a, and -92a are proven to promote GCSCs properties[111]. miR-20a may also be involved in the development of chemoresistance in GCSCs by modulating apoptosis through livin and survivin.

Repair and prevention of DNA damage

The dynamic balance between DNA damage and repair depends on the type of injury and the activity of a variety of repair mechanisms: nucleotide-excision repair (NER), base-excision repair (BER), mismatch repair (MMR), non-homologous end-joining (NHEJ), and homologous recombination (HR) systems. DNA damage-inducing agents are among the most effective treatment regimens in clinical chemotherapy. However, GCSCs can be resistant to DNA damage by drug treatment-induced reactive oxygen species (ROS) scavenging. Gastrointestinal cancer cells with high CD44 expression exhibited an enhanced capacity for GSH synthesis, resulting in defense against ROS[112]. CSC marker ALDH can facilitate detoxification by scavenging of ROS, as well as by producing antioxidant compounds such as NADP[113]. Aldehyde dehydrogenase 3A1 was found to be upregulated in gastric cancer stem-like cells[114]. Moreover, in multiple GC cell lines and hematopoietic malignancies, ALDH is highly expressed in ROS-low cells, and ALDH-high/ROS-low cells may be cancer-initiating cells (CISs)[115-117], which are also called CSCs. These data indicate that ALDH+ GCSCs can enhance the resistance to chemotherapy or radiochemotherapy by reducing the level of ROS and avoiding DNA damage.

Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process of lineage transition whereby epithelial cells lose their epithelial traits and acquire mesenchymal cell phenotypes, with corresponding changes in cell morphology and expression of surface markers[118]. EMT facilitates tumor cell migration, invasion, metastasis, and drug resistance[119]. Several cytokines, chemokines, and growth factors can trigger EMT by activation of a group of EMT-inducing transcription factors (EMT-TFs) such as SNAIL, SLUG, ZEB1/2, and TWIST[120]. EMT is regulated by a wide, complex, interactive molecular network including exogenous inducers, intracellular regulatory miRNA, epigenetic modulators, and cellular signaling pathways including MAPK, ERK, PI3K, SMADs, and Wnt/β-catenin[121].

EMT has been shown to regulate the acquisition of stemness in multiple cancer cells[122] and promote CSC stemness and quiescence that increase drug resistance[123]. EMT could induce CSC characteristics that increase drug resistance through different mechanisms including the hedgehog, Wnt, Notch, and Musashi signaling pathways, as well as the epigenetic regulator Bmi1[124,125].

EMT activation confers drug resistance in CSCs through other mechanisms, including promoting drug efflux by increased levels of ABC pumps or inhibition of cell apoptosis by elevated expression of anti-apoptotic proteins such as Bcl-XL[123-124,126]. Correspondingly, NANOGP8, one of the pseudogenes in the NANOG gene family, is identified to be the main regulator of GCSCs, which can promote EMT/stemness and enhance chemoresistance[127]. NANOGP8 may confer gastric cancer cells with chemoresistance by

upregulation of ABCG2[127]. However, the exact molecular mechanisms responsible for EMT and the resulting drug resistance in GCSCs remain uncertain.

Adaptation to tumor microenvironment

CSCs are found in a specialized tumor microenvironment (TME), known as the niche, which is mainly composed of extracellular matrix (ECM), cancer-associated fibroblasts (CAFs), cancer-associated adipocytes (CAAs), and endothelial, mesenchymal, and immune cells, and those conditions promote CSC adaptation[128-129]. Reciprocal interactions between CSCs and the niche are critical for CSCs to maintain their stemness properties and promote tumor initiation, metastasis, and drug resistance[130].

Increasing evidence highlights that the TME takes part in therapeutic resistance in GCSCs, largely involving CAFs, which remarkably influence the TME via the secretion of various growth factors, cytokines, and chemokines[131]. The main component secreted by CAFs is TGFβ, which induces EMT[132] and promotes the acquisition of GCSC features[133], ultimately leading to drug resistance[134]. Another study showed that CAFs can also promote stemness by the secretion of NRG1, which activates the NF-κB signaling in GC[135]. Moreover, CAFs can induce drug resistance not only by promoting stem-related signaling pathways in GCSCs but also by secreting type I collagen, which contributes to decreasing drug uptake[136]. Additionally, a recent study demonstrated that low expression of gastric CAF-derived SPARC (secreted protein acidic and rich in cysteine) can promote GCSC transformation and 5-FU resistance[137], suggesting that CAF-secreted SPARC may be involved in the regulation of drug resistance of GCSCs. Collectively, this evidence implicates an important role of the TME in the development of drug resistance of GC.

Exosomes

Exosomes (about 30-200 nm) are small extracellular vesicles (EVs) that originate from endosomes and are secreted by live cells into the extracellular space through the fusion of multivesicular bodies (MVBs) with the plasma membrane[138]. They are composed of a transmembrane protein-containing lipid bilayer and cell-state-specific molecules including DNAs, mRNAs, ncRNAs, and proteins in the vesicle lumen. Exosomes, as carriers, mediate cell-to-cell communication and substance exchange via the transfer of donor cell-derived contents to recipient cells[139]. Increasing evidence suggests that tumor-derived exosomes play critical roles in many aspects of cancer, including tumor growth, metastasis, angiogenesis, immunity, and other processes, and can be used as potential diagnostic biomarkers or therapeutic targets for cancer patients[140-142]. Recent studies showed exosomes are associated with the transfer of the drug resistance phenotype, and cancer cells could develop drug resistance after the incorporation of exosomes from drug-resistant cancer cells. Studies indicated exosomal PD-L1 promotes chemoresistance via inducing T cell exhaustion, by which the T cells cannot be reinvigorated by anti-PD-1 treatment[143]. Inhibition of exosomal PD-L1 has also been reported to enhance the efficacy of anti-PD-L1 treatment[143,144]. Furthermore, another study showed chemotherapeutic agents stimulated the secretion of ABCB1-enriched exosomes from drug-resistant cells and increased the transfer of ABCB1 to the recipient cancer cells, thus assisting these sensitive cancer cells in developing the resistant phenotype[145]. More recently, it has been shown that exosomal transference of wild-type EGFR to EGFR-mutated sensitive cancer cells promotes resistance to the mutant-selective EGFR inhibitor osimertinib by activating the MAPK and PI3K/AKT signaling pathways[146]. Thus, exosomes could be novel therapeutic targets, which could overcome resistance to chemotherapeutic drugs[105, 106] or antibody-based approaches[143, 144], and they also might serve as a predictive biomarker for clinical responses to anti-PD-1 therapy[144].

Increasing evidence also highlights that exosomes are involved in the drug resistance of CSCs. The underlying mechanisms are complex, including cell cycle blockage, increased drug efflux, upregulation of detoxifying enzymes, enhanced anti-apoptotic capacity and DNA repair efficiency, inducing EMT process,

and immunosuppression[147,148]. However, the physiological and functional properties of exosomes in GCSCs are still unknown and need further investigation.

Extrachromosomal circular DNA

Extrachromosomal circular DNA (eccDNA) refers to a type of double-stranded circular DNA that originates from but is independent of chromosomes, which is widely present in various eukaryotic cells and can be derived from anywhere in a genome with sizes ranging from hundreds of base pairs (bp) to several megabases (Mb)[149]. According to the size and origin, eccDNAs can be categorized into organelle eccDNAs such as mitochondrial DNAs (mtDNAs) or non-organelle eccDNA such as telomeric circle (t-circles), microDNA (100-400 bp), small polydispersed circular DNA (spcDNA) (100 bp-10 kb), episomes, and double minutes (DMs) (100 kb-3 Mb)[150]. eccDNAs play important roles in gene regulation, sponging of transcription factors, environmental adaptation and evolution, aging, immune response, cell-to-cell communications, and tumor development[151,152].

eccDNAs have been shown to help cancer cells develop drug resistance via various mechanisms [Figure 4]. (A) Amplification of drug target genes: For instance, DMs, which contain the gene coding for dihydrofolate reductase (DHFR), were identified to be amplified and associated with the development of methotrexate (MTX) resistance[153]. (B) Amplification of multidrug resistance (MDR) genes: DMs, bearing the multidrug resistance 1 (MDR1) gene, were amplified in human epidermoid carcinoma cells and caused resistance to various anticancer drugs by upregulation of MDR1[154]. (C) "Hide and seek" mechanism: EGFRvIII, an oncogenic variant, can induce tumor cells to be more sensitive to EGFR tyrosine kinase inhibitor (TKI). Previous studies have demonstrated that erlotinib resistance in glioblastoma is caused by the elimination of DMs containing EGFRvIII[155,156]. However, after erlotinib withdrawal, the mutant EGFR re-emerged on DMs, which induced GBM cells to be re-sensitive to erlotinib treatment[156]. Through this "hide and seek" mechanism, cancer cells can evade drug therapy by dynamic modulation of drug-targeted oncogenes residing on eccDNAs. (D) Increasing intratumoral heterogeneity: eccDNAs can drive heterogeneity among daughter tumor cells, thus inducing these cells to obtain survival advantage under drug pressure[150]. (E) Increasing homologous recombination activity: Homologous recombination is associated with eccDNA biogenesis. Recent studies have shown that homologous recombination activity was increased in DM-carrying MTX-resistant colon cancer cells, whereas inhibition of homologous recombination activity decreased the expression of DM-containing genes and enhanced drug sensitivity in MTX-resistant cells[157].

eccDNAs contribute to a variety of features in cancers and may serve as novel, promising molecular markers to shed new insights into the diagnosis, prognosis, and treatment of cancer patients. However, the functions and underlying mechanisms of eccDNA in CSCs are still unclear and require further exploration.

Helicobacter pylori infection

Helicobacter pylori (*H. pylori*) infection remains a main risk factor in the development of GC. In 1994, *H. pylori* was diagnosed as a Group I carcinogen by the WHO (World Health Organization)[158]. The stem cell hypothesis of cancer formation is that stem or progenitor cells can acquire CSC characteristics, evade homeostatic control, and lead to carcinogenesis. *H. pylori* has been shown to induce EMT and cancer stem cell (CSC)-like properties in gastric epithelial cells[159,160] and gastric cancer cells[161,162]. Data from multiple studies show that *H. pylori* may directly interact with gastric stem/progenitor cells[163-164] or bone marrow-derived cells (BMDCs)[165] to impact the status and properties of these cells, which could be responsible for generating GCSCs. Moreover, *H. pylori* infection can induce inflammation, impact the local microenvironment, and affect gastric stem/progenitor cells and their differentiation by inducing genetic or epigenetic alterations[166-168]. *H. pylori* infection can mediate oncogenic transformation by inducing GCSCs generation or affecting gastric stem/progenitor cells. However, the underlying mechanisms leading to GCSC

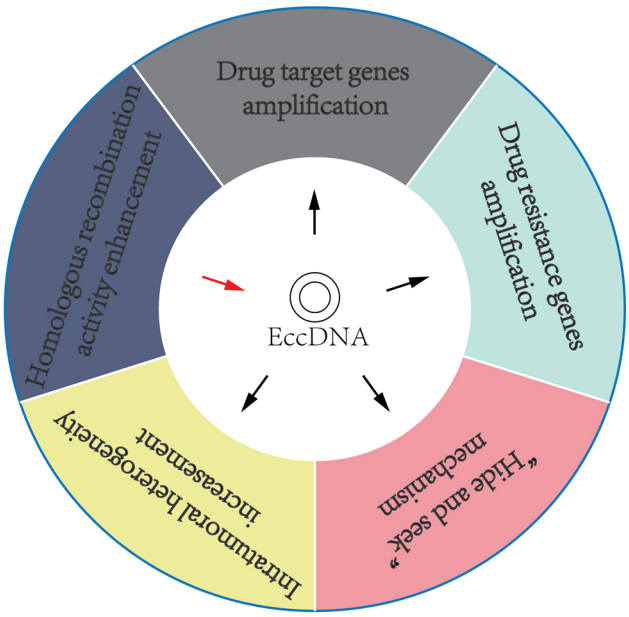

Figure 4. Overview of our current understanding of cancer drug resistance mechanisms induced by eccDNAs.

emergence and the resulting drug resistance in GCSCs in response to *H. pylori* infection are awaiting further investigation.

CONCLUSION

Conventional radio-/chemotherapy provides a limited effect on prolonging the survival of advanced GC patients, and, recently, accumulating evidence shows that GCSCs are resistant to conventional chemotherapy and play a direct role in tumor metastasis and relapse. Based on the extensive evidence presented in this review, it is obvious that GCSCs regulate tumor radio-/chemoresistance via multiple intrinsic and extrinsic mechanisms. This review aims to provide an understanding of the precise mechanisms underlying GCSC resistance to chemotherapeutic drugs. Identifying the molecules and revealing insight into their interaction networks through further investigations may help to discover novel targets of anticancer therapy, develop new therapeutic approaches for the prevention of tumor recurrence and resistance, and increase the lifespan of GC patients.

However, to date, molecular mechanisms of drug resistance in GCSC remain largely unclear. Many aspects are still in need of further clarification: (1) to find more key components or molecules for regulating GCSCs resistance to the anticancer agents; (2) to define the precise molecular mechanisms and clarify how GCSCs coordinate these different, complex molecular pathways to response the chemotherapeutic drugs; and (3) to find the specific GCSC markers related to its response to the anticancer agents, so as to evaluate the effectiveness of different drugs and therapeutic strategies. More importantly, many more need to be proven to be effective in the clinic. We are at the beginning of understanding drug resistance from gastric cells to GCSCs. More basic and clinical studies should be done to increase the knowledge about the mechanisms of drug resistance to improve the outcome of advanced GC patients.

DECLARATIONS

Authors' contributions

Wrote the manuscript: Xiong J, Fu L

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Conflicts of interest
All authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

Original Article

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Butein-instigated miR-186-5p-dependent modulation of TWIST1 affects resistance to cisplatin and bioenergetics of Malignant Pleural Mesothelioma cells

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Abstract

Aim: Malignant pleural mesothelioma is a chemoresistant tumor, and biphasic and sarcomatoid histologies portend the worst prognosis for malignant pleural mesothelioma (MPM) patients. We obtained the microRNA expression profile of three biphasic-sarcomatoid MPM cell lines to identify commonly expressed microRNAs and evaluate the effect of butein, a chemo-sensitizing compound, on this microRNA subset.

Methods: Nanostring-based microRNA profiling and analysis through the ROSALIND platform were employed to identify the commonly modulated microRNAs and their targets. MicroRNA-mimic transfection, Luciferase assay, and Western blotting were employed to show specific perturbation of TWIST1 levels by miR-186-5p. Sphere-forming assays, invasion assay, and metabolic profiling were used to assess the biological consequences of the butein-instigated miR-186-5p-mediated perturbation of TWIST1 levels. TGCA analysis was used to search for the correlation between TWIST1 and miR-186-5p levels in biphasic and epithelioid MPM specimens.

Results: We identified a set of perturbed microRNAs, common to three biphasic/sarcomatoid MPM cell lines, after butein treatment. When focusing on miR-186-5p, we unraveled a butein-ignited and miR-186-5p-mediated modulation of TWIST1 levels which affected the 3D anchorage-independent growth, cisplatin resistance, invasion, and bioenergetics of the MPM cell lines tested. We showed that miR-186-5p and TWIST1 levels are anti-correlated in biphasic MPM specimens from TCGA.

Conclusion: We unraveled a novel mechanism of action of butein, which attenuated the pro-tumorigenic features of MPM at least through a miR-186-5p-TWIST1 axis. We suggest that those activities converge into the chemo-sensitizing effect of this compound and may be of translational relevance.

Keywords: Mesothelioma, butein, miR-186-5p, TWIST1, pithelial-to-mesenchymal transition (EMT), chemoresistance, cancer metabolism, invasion, anchorage-independent growth

INTRODUCTION

Mesothelioma is a neoplastic disease arising from the mesothelial linings of the pleural and peritoneal space. Its pathogenesis and progression envisage genomics alterations and environment-derived chronic inflammation in a complex interplay, with much left to be understood[1,2]. Three main histological presentations characterize malignant pleural mesothelioma (MPM), named epithelioid, biphasic, and sarcomatoid[3]. Rather than being separate entities, these histotypes may represent a structural–functional continuum where epithelial-to-mesenchymal transition (EMT) plays an important role[4]. EMT may correlate with specific pro-tumorigenic features[5], which contribute to the worse prognosis of biphasic and sarcomatoid MPMs. In fact, the expression of EMT genes has prognostic significance in MPM[6]. MPM exhibits long clinical latency and significant resistance to therapy, with the latter impacting only to a limited extent the natural history of the disease. As a result, prognosis for MPM patients results in poor PFS and OS[1,7,8]. The mainstay of MPM treatment is still represented, for first-line or inoperable patients, by cisplatin and antifolate[1,9]. Ex vivo studies have shown that MPM cells are endowed with high resistance to therapy[10]; therefore, attenuating such a process is an unmet need. We and others showed that specific rearrangement of cell subpopulations, sustained by the acquisition of a senescence-associated secretory phenotype (SASP), may underlie the emergence of MPM chemoresistance[11-14]. We found that a naturally occurring compound with pleiotropic functions, butein (20,40,3,4-tetrahydroxychalcone), interfered with the emergence of those chemoresistant aldehyde dehydrogenase-positive ($ALDH^{pos}$) cell subpopulations, by simultaneously blocking NFkB and STAT3 signaling[15,16]. Such interference with the intra-tumor heterogeneity of MPM translates into reduced adaptive stress responses and, ultimately, chemo-sensitizing effects, partially sustained by changes in the gene expression profile of the treated cells[17]. Butein is also known to exert chemo-sensitizing effects via AKT signaling and modulate the MAPK pathway through its antioxidant functions[18-20]. We also recently showed that butein modulates the levels of DNA repair genes, which further contributes to its chemo-sensitizing activity[17].

miRNAs are non-protein-coding single-stranded RNAs[21]. miRNAs bind generally to the 3' untranslated region (3'-UTR) of mRNAs of target genes, thereby functioning as the negative posttranscriptional regulators of gene expression[22]. miRNAs modulate key processes of tumor initiation and progression, ranging from the acquisition of pro-metastatic features to metabolic reprogramming and chemoresistance[23-26].

miR-186-5p is a debated miRNA since its role ranges from oncogenic to a tumor suppressive one, in a cancer-tissue and stage-specific manner[27]. There is an established link between miR-186-5p expression and resistance to therapy including cisplatin, taxol, and methotrexate[28-33]. miR-186-5p was shown to target

twist-related-protein-1 (TWIST1), a key EMT-related transcription factor, in three different settings[28,30,34]. Changes in TWIST1 level could portend a poor prognosis in TCGA cohorts of several cancer settings[6] . TWIST1 modulates aerobic glycolysis in pancreatic cancer cells by increasing the expression of key glycolytic genes, including *HK2* and *PKM2*[35]. Insightfully, the ability of TWIST1 to impinge on EMT and metabolic reprogramming may converge toward the acquisition of chemoresistant phenotype. In fact, EMT is a key process toward the acquisition of chemoresistance[36,37]. TWIST1 is upregulated in MPM tumors and cell lines and may play a role in the development of MPM[6,38]. Further, vaccines against TWIST1 were recently shown to enhance CTLA-4 blockade in experimental mesothelioma immunotherapy approaches[39].

Butein was shown by others and us to be capable of reversing chemoresistance to cisplatin and pemetrexed in MPM[15,16,18]. We hypothesized that butein exerted its chemo-sensitizing effects at least partially through microRNA modulation. Thus, we performed a microRNA expression analysis of three MPM cell lines with sarcomatoid and biphasic originating histotypes and identified 33 microRNAs modulated by butein. Among the identified targets, we focused on the miR-186-5p axis and demonstrated a miR-186-5p-mediated modulation of TWIST1 expression level. We showed that such modulation may explain at least in part the chemo-sensitizing effect of butein, thereby perturbing 3D anchorage-independent growth, cisplatin resistance, invasion, and bioenergetics of the MPM cells.

METHODS

Reagents

Butein ($C_{15}H_{12}O_5$) was purchased from Cayman Chemicals (Ann Harbor, MI, USA) and dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA). Cisplatin (CDDP) was purchased from Selleckchem (Houston, TX, USA).

Cell culture

Cells were cultured in Ham's F12 supplemented with L-glutamine 10% fetal calf serum (Gibco BRL, Grand Island, NY), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The human MPM cell lines MSTO-211H, NCI-H2373, and HP1 were described previously[12]. All cell lines were in-house tested for mycoplasma contamination by using a commercially available PCR-based assay (R&D Systems, Minneapolis, USA).

Cell metabolism

A Seahorse Bioscience XF24 Extracellular Flux Analyzer was used to measure the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). Logarithmically growing mesothelioma cell lines were maintained in normal complete growth media and seeded onto a gelatin-coated 24-well XF Flux Analyzer assay plate at 80,000 cells/well (NCI-H2373) or 40,000 cells/well (HP1 and MSTO.211H) 24 h prior to assay. These seeding numbers were determined based on the doubling time of the mentioned cell lines. Cells were switched to serum-free XF assay media (Seahorse Biosciences, Billerica, MA, USA) with 25 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine and placed in a CO_2-free incubator at least 2 h before the assay. Multiple measurements were obtained at baseline and following injection, sequentially of glucose (10 mM), oligomycin (2 μM), and 2-deoxyglucose (100 mM) (Merck Life Science, Milan, Italy) for ECAR measurement and oligomycin (1 μM), FCCP (0.25 μM), and rotenone (1 μM) (Merck Life Science, Milan, Italy) for OCR measurement. Values are reported as mpH/min for ECAR and pmoles/min for OCR.

Western blotting assay

Whole cell extracts (40-50 μg) from cells or tissues were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad, Hercules, CA, USA). The membranes were blocked and then probed with antibodies against TWIST1 (ab50887) and anti-alpha tubulin (ab176560) as a loading

control (Abcam, Cambridge, MA, USA). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies.

Transfection of mimic-186-5p

The miR-186 mimics (mirVana® miRNA mimic) and its negative control (mirVana™ miRNA Mimic Negative Control) (ctrl) mimics were from ThermoFisher (Waltham, MA, USA). Cells were seeded in 60 mm dishes and transfected (25 nM each) in Ham's F12 with reduced serum [2% fetal bovine serum (FBS)] using the JetPrime reagent (Polyplus Transfection, New York, NY) before being processed for downstream analyses.

Luciferase 3-UTR assays

The TWIST1 3' UTR fragment sequence containing the binding site with miR-186-5p was cloned into the pMIR-reporter vector (Addgene, Cambridge, MA). A quick-change site-directed mutagenesis kit (Stratagene, CA, USA) was used to mutate the miR-186-5p binding site. For reporter assays, the MPM cell lines and the HEK293 cells (as a technical control for higher transfection efficiency) were transiently co-transfected with the TWIST1 3' UTR luciferase vector or mutant 3' UTR with miR-186 mimic or ctrl by JetPrime reagent (Polyplus Transfection, New York, NY). The firefly luciferase activities were measured consecutively using the Luciferase Reporter assay system (Promega, Madison, NJ, USA), according to the manufacturer's protocol. The percentage of luc activity in the cells transfected with miR-186-5p mimics over the cells transfected with the ctrl mimics was reported.

Invasion assay

Cell invasion was assessed using a Matrigel invasion assay. Briefly, diluted Matrigel matrix was carefully added to the center of each Transwell® insert (8 μm PET membrane, Corning, NY, USA) for invasion assays. Cells were starved of serum for 24 h and then seeded in triplicate. Lower chambers contained serum-free medium or medium supplemented with 20% FBS. The inserts were washed twice with PBS1X before fixing and staining in crystal violet solution for 15 min and then air-dried. The invaded and migrated cells were observed and imaged under a microscope. The bound crystal violet was eluted with 33% acetic acid and the eluent absorbance at 590 nm was measured.

Sphere-forming assay

For generating cell spheroids, a variable number of single cells/well were seeded into BIOFLOAT TM 96-well plates (FaCellitate, Germany) in DMEM-F12/1:1 + Glutamax supplemented with B27 (no RA), BSA, bFGF (20 ng/mL), and hEGF (10 ng/mL) (Life Technologies Inc., Grand Island, NY, USA).

Assessing chemo-sensitivity to cisplatin

For the determination of IC_{50}, formed MPM spheroids at Passage 2 were incubated in media with or without the addition of cisplatin (0-100 μM) for 12 h before drug withdrawal. After 72 h, the IC_{50} was defined as the cisplatin dose capable of reducing by 50% the average number of spheroids from four to six independent 96 wells.

Viability assay

Cells were shortly pulsed with butein (10 μM) or ctrl (0.01% DMSO) for 8 h and viability was assessed by flow cytometry-based detection of Sytox-Blue positive cells (Sytox Blue Dead cell stain, Thermo Fisher, CA USA) at 12, 24, 48, 72, and 96 h after drug withdrawal.

RNA expression analysis

Analysis was performed on all samples using the nCounter Analysis System (NanoString Technologies, Seattle, WA, USA) and the nCounter Human v2 miRNA Panel that contains 798 unique miRNA barcodes. Probes for housekeeping genes such as ribosomal protein L10 (RPL10), beta-actin (ACTB), beta-2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ribosomal protein L19 (RPL19) were used as internal controls.

ROSALIND® nanostring miRNA expression analysis

Data were analyzed by ROSALIND® (https://rosalind.bio/), with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA). Normalization, fold changes, and *P*-values were calculated using criteria provided by Nanostring (Seattle, WA, USA). Following background subtraction based on POS_A probe correction factors, normalization was performed in two steps: positive control normalization and codeset normalization. During both steps, the geometric mean of each probeset was used to create a normalization factor. ROSALIND calculated fold changes and *P*-values for comparisons using the *t*-test method. *P*-value adjustment was performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR).

Statistical analysis

Where appropriate, statistical analysis was performed using Student's *t*-test and $P \leq 0.05$ were considered statistically significant. Group analysis was performed by ANOVA and Prism GraphPad software.

Principal component analysis

For creating principal component analysis (PCA) plots, Clustvis was employed https://biit.cs.ut.ee/clustvis.

RESULTS

Butein treatment modulated microRNA expression levels

We performed microRNA profiling of three MPM cell lines: NCI-H2373, HP1, and MSTO-211H [Figure 1]. The three cell lines shared a biphasic (HP1, MSTO211H) and sarcomatoid (NCI-H2373) originating histo-type, both associated with worse prognosis and lower response to therapy. The three MPM cell lines exhibited different microRNA expression patterns, as assessed by PCA [Figure 1A]. We interrogated this microRNA expression profile to identify a set of microRNAs common to all three cell lines, thereby hypothesizing that commonly modulated sets of microRNAs can predict functions common to the biphasic/sarcomatoid cyto-type. We thus identified a limited set of commonly expressed microRNA ($n = 33$, Figure 1B, C and Supplementary Table 1). Butein treatment, executed at non-apoptotic concentrations and schedule of administration[17] [Supplementary Figure 1A], determined a clear perturbation of microRNA levels, as assessed by the PCA analysis [Figure 1B and C]. This revealed a clear separation, on the main PC component (PC1: 81.9%), of the three MPM cell lines as a function of butein treatment [Figure 1D]. When focusing on microRNAs significantly modulated by butein treatment, we observed that microRNA downregulation prevailed after treatment with butein, with a much smaller subset ($n = 6$) of microRNAs upregulated by butein [Figure 1C].

TWIST1 is a miR-186-5p target in butein-treated MPM cells

Among the upregulated microRNAs, we focused on miR-186-5p for its important contribution to EMT and chemoresistance in other cancer settings and because one of its targets, TWIST1, is an important factor in MPM progression[40,41] and a target of butein[16]. To verify that TWIST1 was a target of miR-186-5p, we co-transfected the three MPM cell lines and a non-MPM cell line (HEK293, as a control) with either miR-186-5p mimics or its control (ctrl mimics) [Supplementary Figure 1B] at 25 nM and with expression vectors containing luciferase under the control of the TWIST1 3'-UTR region, wild-type (wt), or mutated (mut)

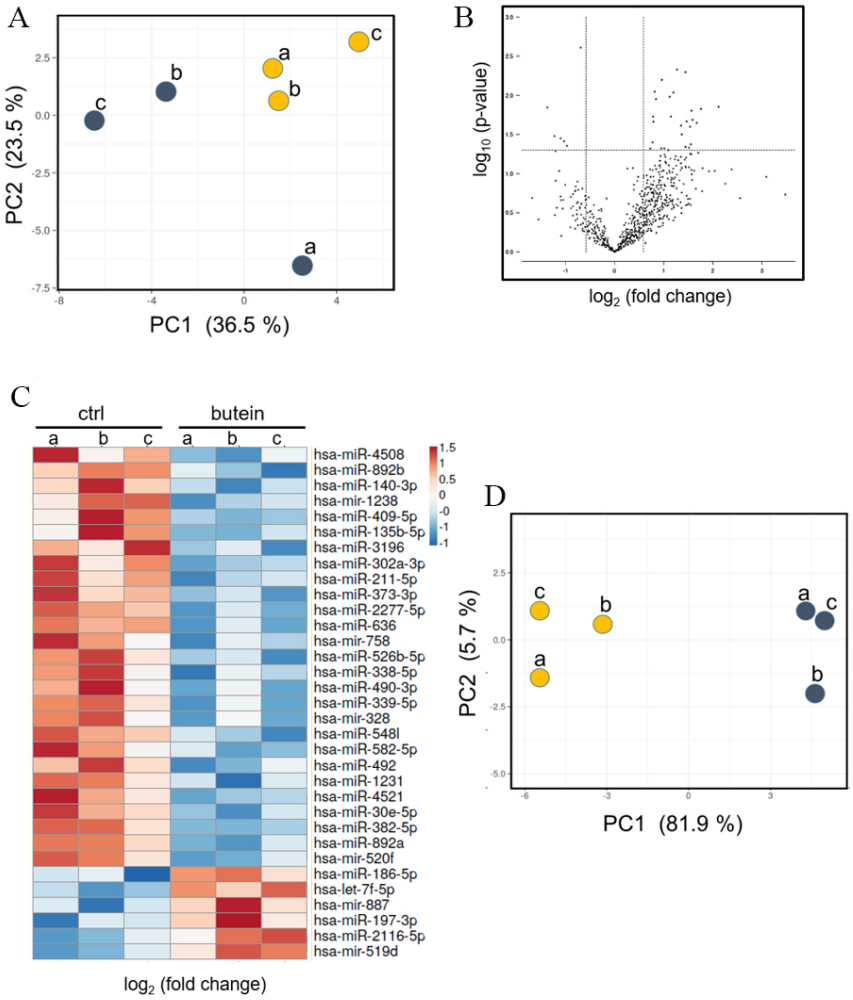

Figure 1. Butein treatment modulated microRNA expression levels. (A) PCA plot of the microRNAs expressed in: HP1 (a); NCI-H2373 (b); and MSTO-211H (c). Blue indicates samples treated with ctrl (DMSO 0.01%), while yellow indicates samples treated with butein for 8 h at 10 μM. (B) Volcano Plot showing the microRNAs commonly modulated in all three MPM cell lines and whose perturbation by butein reached statistical significance. (C) Heatmap showing the normalized levels of the microRNAs common to the three MPM cell lines, treated as in (A). Log2 fold changes are reported. The average of the two experiments is shown. (D) PCA plot showing the distribution of the three MPM cell lines treated as in (A), after selecting the commonly modulated miRNAs.

[Figure 2A, inset]. The luciferase activity in the MPM cells transfected with miR-186-5p mimics was significantly lower than that in the cells with control sequences [Figure 2A]. HEK293 cells exhibited the highest degree of luciferase downregulation, possibly as a consequence of increased transfection efficiency [Figure 2A]. To confirm that the reduced luciferase activity was caused by miR-186-5p binding to the seed site of 3'-UTR, the seed sequence of TWIST1 3'-UTR was mutated in the luciferase reporter construct. No significant changes in the luciferase activity were recorded when the mutated 3'-UTR-luc construct was co-transfected with the miR-186-5p mimics [Figure 2A]. We performed Western blotting experiments in NCI-H2373 cells transfected with either ctrl or miR-186-5p mimics or treated with ctrl (DMSO) or butein. We found that both butein treatment (10 μM for 8 h) and miR-186-5p mimics similarly affected the TWIST1 protein levels, when compared to their respective controls [Figure 2B], and this held true for all three MPM cell lines tested [Figure 2C].

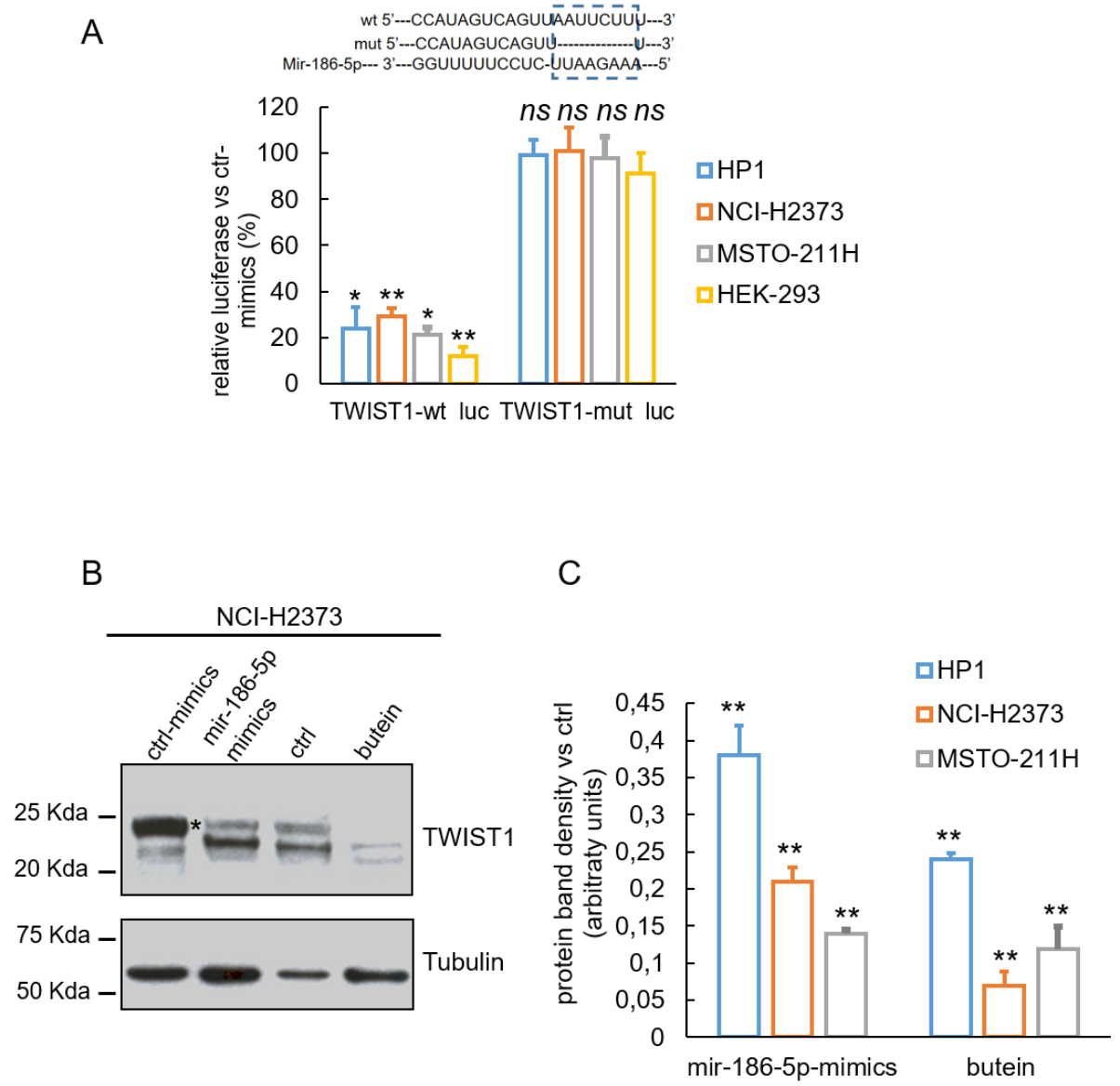

Figure 2. TWIST1 is a miR-186-5p target. Inset: The putative binding site of miR-186-5p within the Twist1 3'-UTR is shown, with the paired sequences of the wt and mutant (mut) constructs generated. (A) The pMIR-Twist1-WT and pMIR-Twist1-MT vectors were transfected into the three MPM cell lines and in HEK293 cells along with miR-186-5p or ctrl mimics and luciferase activity was assessed 48 h later. The percentage of luciferase activity in the cells transfected with miR-186-5p mimics over the cells transfected with the ctrl-mimics is reported. Means ± SD of three replicates are shown. *$P < 0.05$, **$P < 0.01$. (B) Twist1 protein levels in NCI-H2373 cells treated with ctrl or miR-186-5p mimics or butein and ctrl (DMSO 0.01 %) as assessed by Western blotting. Anti-tubulin staining was used as a loading control. Asterisk (*) indicates the TWIST1-specific protein band. (C) Histograms reporting the relative (normalized) protein band intensity of TWIST1 from all three MPM cell lines treated as indicated in (B). The average values of two independent experiments are reported. **$P < 0.01$ (*vs.* ctrl).

Butein-instigated increase of miR-186-5p affected the sphere forming ability and the cisplatin sensitivity of 3D-grown MPM cells

To study the effect of butein-instigated miR-186-5p-dependent TWIST1 modulation on pro-tumorigenic MPM features, we evaluated the effect of the mentioned treatments on the resistance of MPM 3D spheroids to cisplatin. Spheroid cultures are enriched for the expression of EMT factors and may represent a suitable system to study chemoresistance[12,42,43]. MPM cells were grown as 3D spheroids after seeding in anchorage-

independent, quasi-clonal densities, and sphere forming efficiency (SFE) was evaluated for saline- and cisplatin-treated spheroids after a 12 h pulse of the drug at IC_{25} and IC_{50} doses (empirically determined for each cell line, Supplementary Figure 2A) [Figure 3]. Evaluation of the size, appearance, and number of the formed spheroids after an additional 48 h revealed a clear change in the morphology of the cisplatin- *vs.* ctrl-treated spheroids (Figure 3A, top and bottom, respectively), with the cisplatin treated ones being much less compact and rounded and significantly reduced in number [Figure 3A]. Butein treatment (10 μM, 8 h) [Supplementary Figure 2B] induced spheroid disaggregation in the ctrl-treated cultures, which was even more dramatic in the samples co-treated with cisplatin, suggesting an effect on cisplatin sensitivity of the spheroids [Figure 3A and B]. Transfection of the miR-186-5p constructs induced very similar effects when compared to butein, consisting of spheroid disaggregation in ctrl-treated samples, which was more prominent in the cisplatin treated ones [Figure 3A and B]. On the other hand, overexpression of TWIST1 induced the formation of round and compact spheroids, which were significantly resistant to cisplatin treatment, as evaluated by morphology and number [Figure 3A and B]. We observed similar effects when challenging the spheroids obtained from MSTO-211H and HP-1 MPM cell lines with a similar treatment scheme [Supplementary Figure 3A and B]. Thus, butein could both attenuate the SFE and reduce the resistance of MPM spheroids to cisplatin, at least partially through a miR-186-5p-mediated downregulation of TWIST1.

TWIST1 is a critical effector of miR-186-5p-mediated inhibition of invasion

Next, we evaluated the effect of butein-instigated miR-186-5p-dependent TWIST1 modulation on the invasive properties of NCI-H2373 cells [Figure 4]. Staining of the cells which invaded the Matrigel-coated surface and evaluation of the optical density in time revealed that, when compared to ctrl (DMSO), butein strongly affected NCI-2373 invasion [Figure 4A], and this inhibition appeared as early as 24 h after cell treatment and lasted over time [Figure 4B]. Overexpression of TWIST1 strongly increased the invasive ability of the NCI-H2373 cells, as compared to the cells transfected with the vector alone [Figure 4A], and this was maximal at 72 h after the transfection [Figure 4B]. These results were consistent in MSTO-211H [Supplementary Figure 4], while the HP1 cells did not show relevant invasion at steady state and were not tested further (data not shown). Thus, butein could inhibit the invasion of MPM cells at least partially through a miR-186-5p-mediated downregulation of TWIST1.

A butein-instigated increase of miR-186-5p modulated oxidative mitochondrial respiration and glycolytic activity of MPM cells

Next, we evaluated the effect of butein, miR-186-5p, and TWIST1 on the bioenergetics of the NCI-H2373 cells. We evaluated the basal ECAR and OCR of NCI-H2373 cells treated with ctrl or butein and transfected with ctrl or miR-186-5p mimics or TWIST1 [Figure 5A and B]. We found that butein treatment and, similarly, miR-186-5p mimics reduced the glycolytic flux of the NCI-H2373 cells [Figure 5A]. Both butein and miR-186-5p mimics reduced the OCR of NCI-H2373 [Figure 5B]. However, butein treatment affected the OCR rate to a larger extent than did the miR-186-5p mimics, suggesting that the deeper OCR inhibition by butein may involve additional mechanisms. TWIST1 overexpression did not significantly affect the OCR of NCI-H2373 cells ($P = 0.07$) while readily increasing their glycolytic flux [Figure 5A and B].

We extended our observations to the additional MPM cell lines MSTO-211H and HP1 [Supplementary Figure 5A and B]. This revealed that all three cell lines exhibited a significantly different metabolic profile, with NCI-H2373 being more reliant on mitochondrial oxidative respiration and MSTO-211H being more glycolytic. HP1 showed an intermediate metabolic profile [Supplementary Figure 5A and B]. Despite those differences, evaluation of the basal OCR and ECAR revealed that, in MSTO-211H and HP1 cells as well, butein and miR-186-5p mimic acted very similarly by reducing the glycolytic flux, with TWIST1 enhancing the latter in an opposite way. The OCR was affected to a larger extent by butein than by miR-186-5p mimics

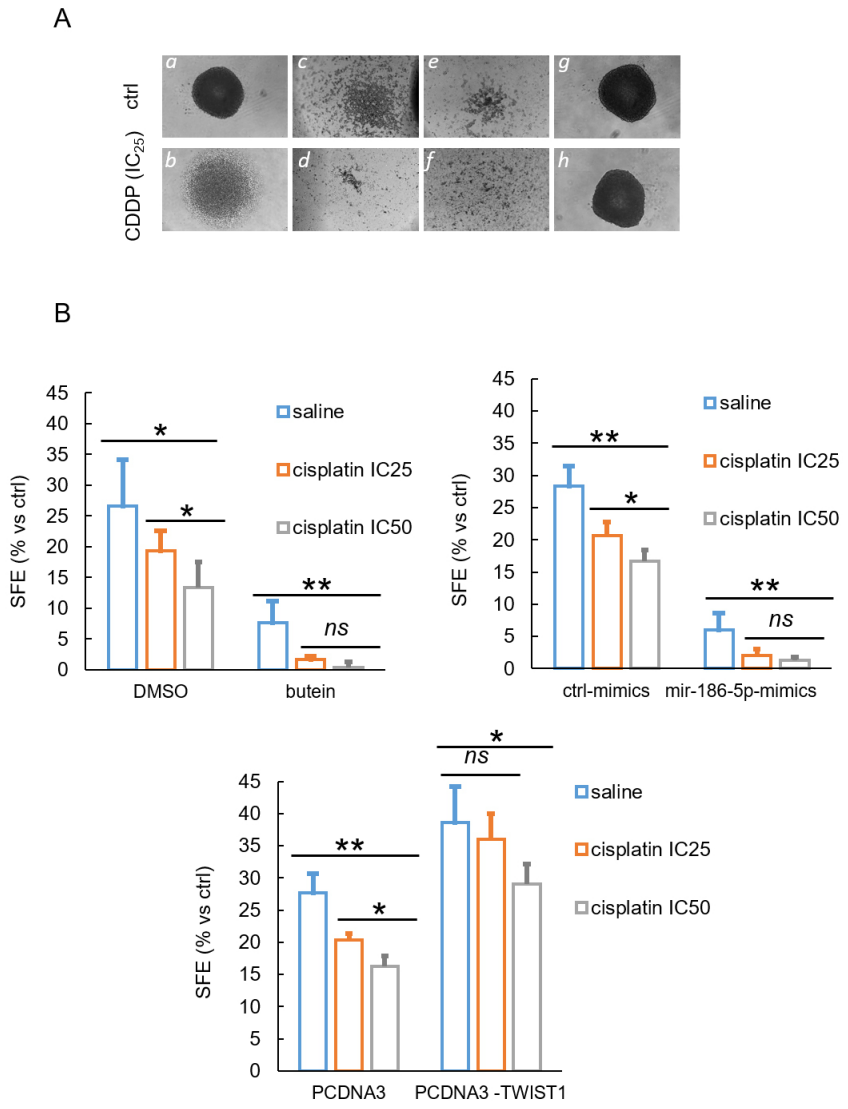

Figure 3. Butein-instigated increase of miR-186-5p affected the sphere forming ability and the cisplatin sensitivity of 3D-grown NCI-H2373 cells. (A) Spheroid formation assay: NCI-H2373 cells, treated with ctrl-(DMSO 0.01%) (*a, b*) or butein (10 μM, 8 h) (*c, d*), transfected with miR-186-5p mimic (*e, f*), or with PCDNA3-TWIST1(*g, h*), were clonally seeded and allowed to form spheroids for 48 h. After that, ctrl (saline: *a, c, e, g*) or cisplatin (*b, d, f, h*) was added at the IC_{25}. Representative micrographs of the formed spheroids (on Day 2 after cisplatin or saline treatment). Scale bar, 200 μm. (B) Histograms showing quantitation of the SFE from NCI-H2373 cells treated with saline or cisplatin at IC_{25} and IC_{50}, respectively, and counted on Day 7 after treatment started. The percentage is relative to the control sample within the group except when otherwise indicated. Asterisk indicate statistical significance as follows: *$P < 0.05$; **$P < 0.01$; ns: not significant ($P > 0.05$). The average of 3 experiments is shown.

[Supplementary Figure 5C and D]. Overexpression of TWIST1 significantly reduced the OCR in MSTO-211H and HP1 cells ($P < 0.05$), possibly as a consequence of the increased glycolytic flux following TWIST1 overexpression [Supplementary Figure 5D]. Those changes were evident by evaluating the OCR/ECAR ratio for all three cell lines [Figure 5C].

Altogether, we found that butein treatment and the transfection of miR-186-5p mimics exerted very similar effects on the 3D anchorage-independent growth, cisplatin resistance, invasion, and bioenergetics metabolism of the three MPM cell lines tested. Conversely, overexpression of TWIST1 induced rather

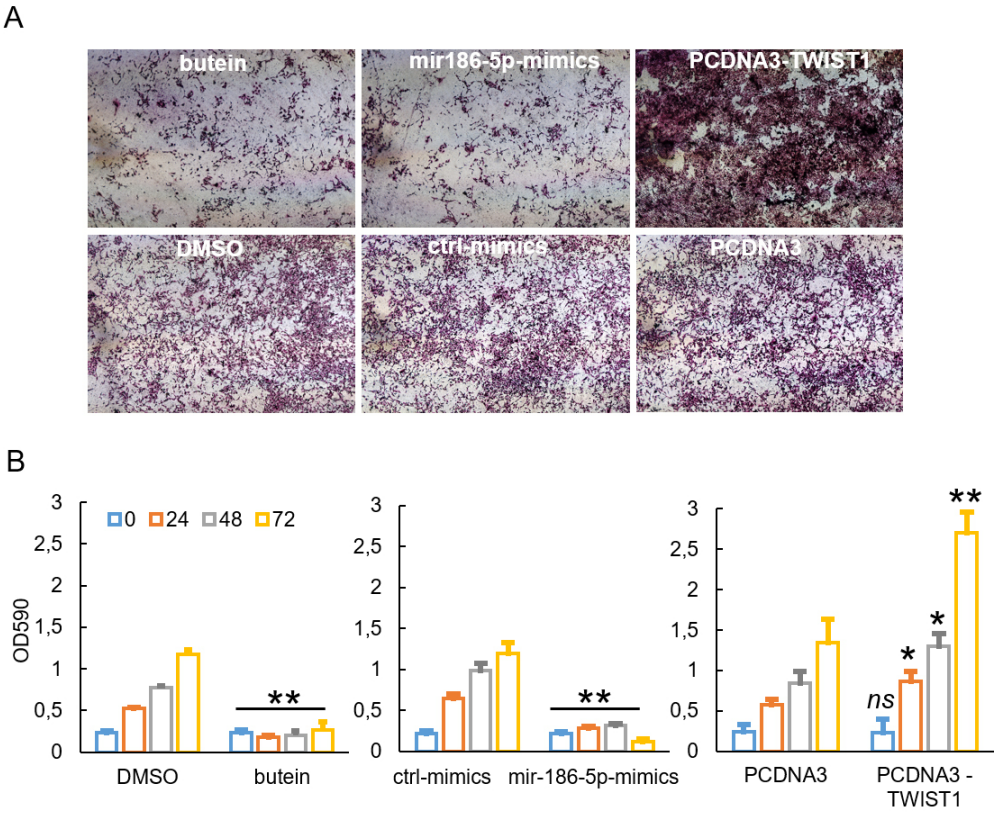

Figure 4. Butein-instigated miR-186-5p-mediated inhibition of TWIST1 attenuated invasion of NCI-H2373 cells. (A) Representative bright-field images of Transwell invasion assay inserts 48 h after seeding of the NCI-H2373 cells. Cells were stained with crystal violet. (B) Histograms showing quantitation of the migrated NCI-H2373 cells treated as in (A) and counted at 24, 48, and 72 h after treatment started. The bound crystal violet was eluted and the absorbance at 590 nm was measured using a plate reader. The average of three experiments is shown. The percentage is relative to the relative control group except where otherwise indicated. Asterisk indicate statistical significance as follows: *$P < 0.05$; **$P < 0.01$; ns: not significant ($P > 0.05$).

opposite effects by enhancing all the mentioned pro-tumorigenic properties.

miR-186-5p and TWIST1 exhibited opposite behavior in biphasic MPM specimens

Finally, we explored the connection between miR-186-5p and TWIST1 in a more clinically relevant setting. When searching for a correlation between miR-186-5p and TWIST1 mRNA levels in the TGCA database, no significant correlation was observed between miR-186-5p and TWIST1 mRNA levels in MPMs (Spearman rho = -0.1936, $P = 0.0748$). However, we found a trend, for biphasic mesotheliomas ($n = 21$) toward exhibiting lower miR-186-5p ($0.05 < P < 0.10$) and higher TWIST1 expression, as compared to epithelioid mesotheliomas ($n = 58$) [Figure 6A and B].

DISCUSSION

We addressed the microRNA profile of three mesothelioma cell lines. We chose to profile biphasic/sarcomatoid cell lines because the originating histotype of those cell lines is linked to a worse prognosis and fewer data are available when compared to the epithelioid histotype MPMs[44]. We uncovered a set of microRNAs commonly and significantly modulated in the mentioned cell lines. We found miR-186-5p as significantly and highly modulated by butein treatment. This focused our attention, given the involvement of one of the miR-186-5p targets, TWIST1, on both the biology of MPM[38,40] and the possibility of explaining the mechanisms behind the chemo-sensitizing effects of butein[16]. This helped us delineate a

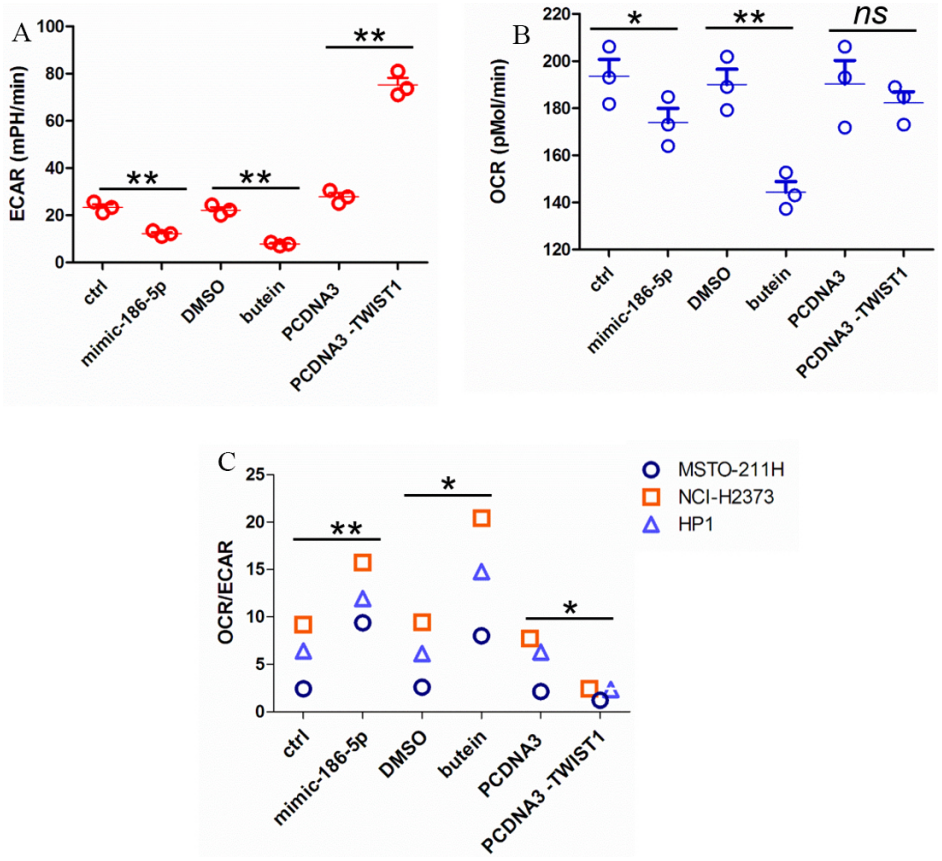

Figure 5. A butein-instigated increase of miR-186-5p modulated oxidative mitochondrial respiration and glycolytic activity of MPM cells. (A, B) Basal ECAR and OCR measured in NCI-H2373 cells treated or transfected as indicated. Values are reported as mpH/min for ECAR and pmoles/min for OCR Results are shown as the mean ± SEM of three independent experiments. Asterisk indicate statistical significance as follows: **P* < 0.05, ***P* < 0.01). (C) OCR to ECAR ratio assessed for all three MPM cell lines of this study.

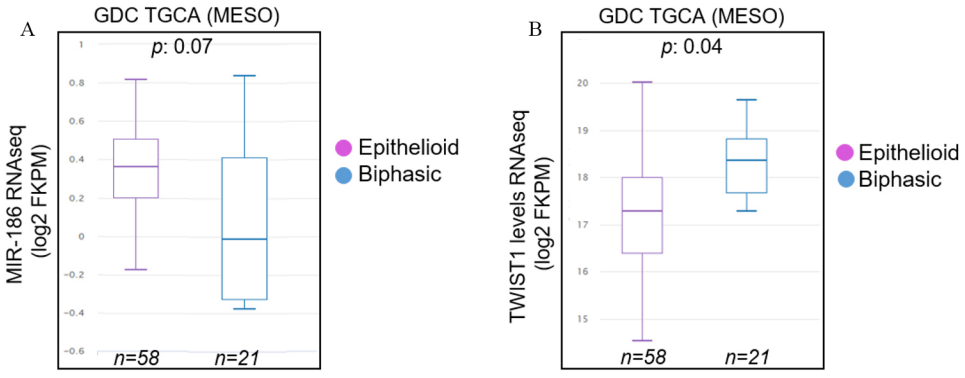

Figure 6. miR-186-5p and TWIST1 anti-correlated in biphasic MPM specimens. (A) Box plot reporting the levels of miR-186-5p assessed by RNAseq and expressed as Log2 (FKPM) in biphasic (*n* = 21) *vs.* epithelioid (*n* = 58) MPM specimens. (B) Box plot reporting the levels of TWIST1 mRNA assessed and reported as in (A) from the same MPM specimens. *P*-values are reported above each graph.

butein-instigated modulation of TWIST1 by miR-186-5p which impinges on the invasion, 3D growth, chemoresistance, and metabolic features of the MPM cells.

EMT is an important feature of MPM, since mesothelial cells show a proclivity to undergo EMT even in pathophysiological conditions, such as peritoneal dialysis[45,46]. We and others showed that EMT sustains and accompanies a therapy-induced SASP which fuels the resistance of MPM cells to cisplatin and pemetrexed[11,14]. More recently, an EMT-omic signature emerged as s distinct prognostic trait of MPM[47] and as a determinant of anti-CTLA-4 immuno-response[11,14,39]. TWIST1, being a key EMT player and a target of microRNA modulation by butein, may well find a place within the chemo-sensitizing actions of this versatile compound. In fact, we showed that, besides the effect on cell invasion and 3D growth, butein-modulated TWIST1 affects cisplatin resistance. A similar effect has recently been shown in epithelial ovarian cancer cells, where TWIST1 delineates a chemoresistant ovarian cancer phenotype[48-50].

In addition to the mentioned anticancer effects, we found that the butein-miR-186-5p axis modulated MPM cell bioenergetics, by reducing glycolytic processing and mitochondrial respiration in all 3 cell lines tested. The degree of metabolic perturbation was similar in all three cell lines but varied in magnitude, according to differences in the three cell lines, already at steady state [Supplementary Figure 5]. Our investigation was limited to assessing ECAR and OCR rates. However, we, by using a more comprehensive metabolic assessment, and others, in different experimental settings[51], showed how specific lipid species may mediate chemoresistance of MPM cells by activating NFkB signaling[12]. Even if there was no demonstration that TWIST1 modulation may directly affect the release of signaling lipids, the fact that butein, TWIST1, and miR-186-5p are involved in chemoresistance and the high degree of connection between metabolic pathways suggests that this may be the case and prompts future detailed investigation.

Here, we identified a novel action of butein, which is the microRNA modulation. Such findings are in line with what we and others showed on the anticancer action of butein, i.e., that it inhibits migration, invasion, clonogenicity, and resistance of the cells to chemo-therapeutics[18]. The effect of butein on TWIST1 matches what is known of the ability of TWIST1 to impinge on AKT signaling and drive resistance to cisplatin[50]. However, this may not be the only mechanism for butein: for example, target prediction of the butein-modulated microRNAs suggested that additional targets may mediate the chemo-sensitization effect of butein (data not shown). In line with this, the effect of butein treatment on the OCR was similar but stronger when compared to that of the miR-186-5p mimic [Figure 5B]. Butein has pleiotropic, metabolic effects including modulation of lipid biosynthesis through NFkB/STAT3 inhibition and TGF-beta-PPARγ interference[52] and HMOX1 induction[53]. Therefore, there is the possibility that butein affects OCR through miR-186-5p-independent mechanisms. Thus, engagement of additional microRNA-target modules and a broader metabolic action may also explain the more profound effects of butein on the OCR of the MPM cell lines when compared to miR-186-5p mimics alone [Figure 5B]. On the other hand, gene expression profiling has already shown how butein may modulate DNA damage associated and DNA repair pathways[17], and it is very likely that several mechanisms converge onto the anticancer action of butein, possibly with different kinetics in time and with tumor-stage specificity *in vivo*.

An unsolved question within this work is how butein may modulate the levels of miR-186-5p. There is some indication that a transcriptional mechanism may be responsible for this. Resistin (RETN) is a proinflammatory cytokine secreted from adipocytes and monocytes[54]. In addition to its pivotal role in inflammation-related diseases, RETN was shown to suppress the miR-186-5p levels, thereby contributing to cancer resistance in ovarian cancer[55] and facilitating VEGF-C-associated lymphangiogenesis in human chondrosarcoma cells[56]. RETN expression is downregulated by an NFkB-mediated transcriptional mechanism in human monocytes[57]. Relevant to this, induction of RETN after butein treatment was recently shown in 3T3 mouse preadipocytes[53]. It is possible that butein, as being a known NFkB inhibitor, modulates RETN levels, thereby attenuating the resistin-mediated downregulation of miR-186-5p.

One limitation of this study is that we did not investigate whether the modulation of miR-186-5p by butein takes place in specific cell subpopulations. In fact, we showed that butein exerts differential effects on FACS sorted, chemoresistant MPM cell subpopulations, such as the ALDH^{bright} MPM cells[17]. Another limit of this study is that we did not address mechanistically how TWIST1 downregulation by butein may affect the pro-tumorigenic program. Since TWIST1 functionally interacts with AKT, and we and others showed that AKT is a downstream collector of survival signaling in pemetrexed-treated MPM cells[50,58], it is possible that a TWIST1-AKT axis may be effective even in this experimental setting.

Finally, we did not make use in this work of primary MPM specimens, which may represent a more clinically relevant experimental setting. However, knowledge in the literature exists that TWIST1 was significantly increased in a gradient fashion when analyzing epithelioid, biphasic, and sarcomatoid primary MPMs[40]. We expanded on these data by observing that TWIST1 mRNA levels are anti-correlated with miR-186-5p levels in TGCA biphasic but not in epithelioid MPM specimens [Figure 6A and B].

DECLARATIONS

Authors' contributions

Performed the experiments, analyzed all the data and amended the revised version: Cioce M, Rutigliano D
Contributed reagents/materials/analysis tools and critically revised the manuscript: Puglielli A, Fazio VM
Conceived and designed the experiments: Cioce M
Read and approved the final manuscript: Rutigliano D, Puglielli A, Fazio VM, Cioce M

Availability of data and materials

The datasets and certain material used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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A nano-enhanced vaccine for metastatic melanoma immunotherapy

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Abstract

Aim: Despite the huge advancements in cancer therapies and treatments over the past decade, most patients with metastasized melanoma still die from the disease. This poor prognosis largely results from resistance to conventional chemotherapies and other cytotoxic drugs. We have previously identified 6 antigenic peptides derived from melanomas that have proven efficacious for activating $CD4^+$ T cells in clinical trials for melanoma. Our aim was to improve pharmacodynamics, pharmacokinetic and toxicological parameters by individually encapsulating each of the 6 melanoma helper peptides within their own immunogenic nanoliposomes.

Methods: We modified these liposomes as necessary to account for differences in the peptides' chemical properties, resulting in 3 distinct formulations. To further enhance immunogenicity, we also incorporated KDO2, a TLR4 agonist, into the lipid bilayer of all nanoliposome formulations. We then conducted *in vivo* imaging studies in mice and *ex vivo* cell studies from 2 patient samples who both strongly expressed one of the identified peptides.

Results: We demonstrate that these liposomes, loaded with the different melanoma helper peptides, can be readily mixed together and simultaneously delivered without toxicity *in vivo*. These liposomes are capable of being diffused to the secondary lymphoid organs very quickly and for at least 6 days. In addition, we show that these immunogenic liposomes enhance immune responses to specific peptides *ex vivo*.

Conclusion: Lipid-based delivery systems, including nanoliposomes and lipid nanoparticles, have now been validated for pharmacological (small molecules, bioactive lipids) and molecular (mRNA, siRNA) therapeutic approaches. However, the utility of these formulations as cancer vaccines, delivering antigenic peptides, has not yet achieved the same degree of commercial success. Here, we describe the novel and successful development of a nanoliposome-based cancer vaccine for melanoma. These vaccines help to circumvent drug resistance by increasing a patient's T cell response, making them more susceptible to checkpoint blockade therapy.

Keywords: Nanoliposomes, nanoscale drug delivery, cancer vaccines, metastasized melanoma, peptides, melanoma drug resistance

INTRODUCTION

Melanoma of the skin is the 5th most common cancer for both males and females in the United States.[1], and currently, only complete surgical removal is considered curative. In advanced malignant and metastasized stages of melanoma, where surgery alone cannot offer remission, the prognosis is extremely poor. This is because human melanoma is particularly resistant to conventional chemotherapies and other cytotoxic drugs[2-4], and this resistance has primarily been linked to dysregulations in apoptosis[2].

Recent progress in cancer immunotherapy has prolonged median survival for metastatic melanoma from about nine months to about 3 years, but most patients with metastatic melanoma still do not survive[5-7]. A lack of pre-existing T cell immunity to cancer antigens is commonly a root cause of failure. In addition, both intrinsic and adaptive resistance to immunotherapies in melanoma has been observed[4]. Therefore, there is a significant need for new therapies to prevent melanoma recurrence by enhancing immune responses to melanoma. Cancer vaccines offer the promise to induce immune responses to cancer when spontaneous antitumor immunity is absent or weak. The effectiveness of immune checkpoint blockade therapy demonstrates that melanoma cells express antigens that can be recognized by CD4 and CD8 T cells, and also that T cell responses to those antigens can mediate tumor regression, when tumor-associated immune dysfunction is abrogated. There has been recent enthusiasm for approaches targeting antigens created by spontaneous mutations (mutated neoantigens)[8]; however, recent data highlight the value of shared melanocytic antigens as relevant targets[9], and other data highlight the value of vaccinating against shared cancer-testis antigens[10].

Data from our group and others have highlighted the pivotal role of $CD4^+$ (helper) T cells in cancer immunity and cancer control[11-14]. We have previously developed a multi-peptide melanoma vaccine consisting of 6 melanoma helper peptides (6MHP) designed to induce melanoma-reactive $CD4^+$ T cells, and found that they are immunogenic in humans, inducing objective clinical responses and very high rates of 5-year survival. Additionally, we found that the immune responses are enhanced when simultaneously injecting a toll-like receptor 3 (TLR3) agonist with the vaccine[15]. With a different peptide vaccine, we also

found that agonists for TLR4 are useful vaccine adjuvants alone or with incomplete Freund's adjuvant[16]. However, these responses are still modest in absolute magnitude, even with strong adjuvants, and even the best responses usually represent less than 1% of circulating $CD4^+$ T cells. A major goal of current cancer vaccines is to induce stronger and more durable T cell responses, but optimal vaccine strategies in humans have not yet been defined.

Most cancer vaccines contain antigens plus vaccine adjuvants. However, when they are co-administered as soluble agents, there is no assurance that the antigens and the adjuvants will be delivered to the same antigen-presenting cell. Nanoliposomes provide a way to deliver antigens to phagocytic antigen-presenting cells, especially dendritic cells, while simultaneously delivering vaccine adjuvants to the same antigen-presenting cells. The use of nanoliposomes to deliver cancer therapeutics offers significant promise and has had several clinical successes[17]. Indeed, many vaccines benefit from lipid-based delivery. Of particular note are the recently authorized COVID-19 vaccines, which utilize lipid nanoparticles containing antigen-encoding RNA[18,19]. Our team has been successful in the use of nanotechnologies, including nanoliposomes, for a host of different therapies[20-25]. Nanoliposomes have the capability of altering the pharmacokinetic properties of antigens and immunomodulators/adjuvants, with a marked reduction in off-target toxicity, protection of the therapeutic from degradation, along with more specific targeting to antigen-presenting cells[26]. A theoretical benefit of using a nanoliposome vaccine over merely injecting antigens and adjuvants individually is that the nanoliposome ensures all adjuvants circulate together and are delivered to the antigen-presenting cells at the same time. Furthermore, nanoliposomes are readily taken up by the antigen-presenting dendritic cells (DCs)[27-30]; therefore, having a TLR agonist incorporated into the liposome should provide increased $CD4^+$ T cell activation. Taken together, nanoliposomes are ideal nano-carriers and delivery agents for emerging cancer vaccines due to their versatility, ease of modification, and ability to deliver several adjuvants simultaneously[31].

As described above, one of the advantages of liposomal delivery is the ability to incorporate vaccine adjuvants into the peptide delivery vehicle. We have chosen to use the toll-like-receptor 4 (TLR4) agonist, KDO2-lipid A (herein KDO2, but also often referred to as KLA). KDO2 is a synthetic and homogenous form of Lipid A, an essential component of lipopolysaccharides (LPS) in Gram-negative bacteria[32]. KDO2 stimulates potent and reproducible host immune responses through the complex of TLR4 and myeloid differentiation protein 2[32-34]. In previous studies, we have used naturally-derived LPS as a TLR4 agonist[16], and others have used KDO2 and other lipid A derivatives in several cancer immunotherapies[35-38]; thus, it is an appropriate choice of agonist for this study. Here, we want to improve pharmacodynamics, pharmacokinetic and toxicological profiles by incorporating KDO2 directly into the delivery system to enhance $CD4^+$ T cells via TLR4. Thus, we hypothesized that (1) we could develop stable KDO2-containing nanoliposomes for the encapsulation of each of the 6 melanoma helper peptides identified previously by our team; and (2) these nanoliposomes would trigger an increased $CD4^+$ T-cell response over and above delivery of the peptide(s) or TLR4 agonist alone.

METHODS

Encapsulation of melanoma peptides within nanoliposomes

The 6 melanoma peptides that induce melanoma-reactive $CD4^+$ T cells in patients, as well as their protein epitope and ideal pH range for solubility, are provided in Table 1. We initially chose a neutral formulation to encapsulate all peptides and then, through an iterative process, modified formulations to optimize encapsulation efficiencies for several of the peptides. Through this process, we identified 3 different nanoliposome formulations (neutral, cationic and anionic) suitable for distinct peptides. However, all of our formulations contained the same base lipid components, including 1,2-distearoyl-sn-glycero-3-

Table 1. Amino acid sequence of the 6 melanoma helper peptides along with their abbreviation, Epitope (the sequence of amino acids recognized by the T cell receptor, which defines the specificity of the response) and ideal pH range required for stability and dissolution

Amino acid sequence (letter = 1 amino acid)	Abbreviation	Epitope (protein, residue numbers)	Ideal pH range
AQNILLSNAPLGPQFP	AQN	Tyrosinase $_{56-70}$	8.5-9.0
WNRQLYPEWTEAQRLD	WNR	gp100 $_{44-59}$	7.0-8.0
LLKYRAREPVTKAE	LLK	MAGE-1,2,3,6 $_{121-134}$	6.0-8.0
FLLHHAFVDSIFEQWLQRHRP	FLL	Tyrosinase $_{386-406}$	6.0-8.0
RNGYRALMDKSLHVGTQCALTRR	RNG	Melan-A/MART-1 $_{51-73}$	6.0-8.0
TSYVKVLHHMVKISG	TSY	MAGE-3 $_{281-295}$	5.0-5.5

phosphocholine (DSPC); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000PE); KDO2; cholesterol; and a fluorophore [either rhodamine or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD)]. Each formulation contained DSPC and DOPE at a 2.14:1 molar ratio to form a stable, spherical nanoparticle. In addition, cholesterol at a 30 molar percent was incorporated to increase rigidity and reduce leakiness. PEG(2000)-PE at 2.5 molar percent was incorporated for biological stability, KDO2 was added to all formulations at 0.1 molar percent, and a fluorescent probe (rhodamine or DiD) was added at 0.2 molar percent for subsequent biodistribution imaging studies. Keeping the molar ratios of these key components constant, we incorporated hexadecyl phosphate (DHP) at 10 molar percent for the anionic formulation and positively charged lipid 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) at 7 molar percent for the cationic formulation. All lipid components were dissolved in chloroform and mixed in the ratios shown in table 2. The lipid mixtures were then dried down in a nitrogen blower for roughly 2 h until all of the chloroform was evaporated. Then, the peptide solutions (or 1× PBS for the "ghost" formulation) were added to the dried down lipids; the tubes were then vortexed and placed in a heat shaker at 60 °C for 2 h, followed by sonication in a 60 °C sonic bath for roughly five minutes. After sonication, the liposomes were extruded through a 100-nanometer pore membrane eleven times to create uniformly sized liposomes. The extruded liposomes were subsequently run through a Sepharose gel bead column to separate the liposomal drugs from the free drug. The solution was analyzed using dynamic light scattering (DLS) (polydispersity, an average hydrodynamic diameter of particles in solution) and mass spectrometry.

To further ensure optimal encapsulation efficiency of the MHPs, we utilized distinct dissolution buffers tailored to each peptide's optimal pH range and previously optimized for *in vivo* injection of the free peptides into patients enrolled in our earlier clinical studies. Specifically, for the neutral peptide formulations, 1.33 mg/mL sodium bicarbonate ($NaHCO_3$) in a 1:2 solution of lactated Ringer's solution (LR) and water, respectively (B1); for the cationic formulation, 5 mg/mL $NaHCO_3$ in water (B2); for the WNR anionic formulation, 1 mg/mL $NaHCO_3$ in water (B3); and for the TSYVKVLHHMVKISG (TSY) anionic formulation, 1:9 ratio of 2-(N-morpholino) methanesulfonic acid (MES) buffer and water (B4).

Liposomal stability and peptide release studies were conducted by storing the liposomes in different conditions and media. We monitored the liposomes over a 5-week period in both 1X PBS and 10% fetal bovine serum, under refrigerated, room temperature, and body temperature storage conditions. We ran DLS studies, as well as centrifugation followed by mass spectrometry of the supernatant and reconstituted liposome solutions to monitor liposome stability and peptide release, respectively.

In vivo murine studies

A 6-day study was performed to determine the biodistribution of the fluorescent nanoliposomes, utilizing daily live-mouse imaging with an *in vivo* imaging system (IVIS). For this study, four mice received a DiD fluorescently labeled 6MHP-KDO2-nanoliposome injection. Two mice were injected subcutaneously (SQ) into either flank and 2 mice were injected intravenously (IV). Each peptide nanoliposome had been previously formulated and stored separately. On the day of the experiment, all 6 formulations were mixed together in ratios that contained 0.4 μg of each peptide. The mice were administered isoflurane anesthesia. On day zero, once asleep, the mice were injected with the 6MHP-KDO2-nanoliposome mixture and then transferred immediately to the IVIS instrument. Imaging was conducted within 30 s (or less) of injection. For IVIS® Spectrum Image collection and processing, tomographic fluorescent images were collected of all mice using epi-illumination on a Caliper IVIS® Spectrum scanner following anesthetization with inhaled isoflurane inside a conduction chamber. Live imaging was then performed daily for 6 days. On the 6th day, the mice were euthanized, and their organs were harvested for follow-up *ex vivo* studies. The organs selected for harvest included the lungs, liver, spleen, kidneys, and lymph nodes. Organs from the *in vivo* study were transferred to 24-well plates, and fluorescent imaging data were collected on the IVIS® Spectrum Image system for qualitative determination of biodistribution. Fluorescent image collection used Living Image® software by Caliper Life Sciences. Image processing was done by importing Living Image® files into Aura imaging software by spectral instruments imaging, and then performing region of interest (ROI) measurements to quantify the fluorescence emitted from the mice. The fluorescent light images were collected in the same manner for the harvested organs following the live animal imaging once the mice were sacrificed. The measurement for fluorescence is the mean radiant efficiency within the drawn ROI. Radiant efficiency describes the fluorescent energy emitted from the specimen as a fraction of the excitation fluorescent radiation released by the scanner and incident upon the specimen. Mean radiant efficiency is unitless and is used so that comparisons may be made among mice and harvested organs of different sizes, and consequently slightly differing areas within ROIs. ROIs were drawn as subject ROIs according to the Living Imaging® software user manual (Caliper Life Sciences, 2012).

Ex vivo lymphocyte stimulation using immunogenic TSY encapsulated nanoliposomes

Freshly obtained (cryopreserved) peripheral blood mononuclear cells (PBMC) and lymphocytes from the sentinel immunized nodes (SIN) of 2 patient donors (under IRB protocols, clinical trial Mel41 (NCT00089219; IRB #10464)[39] were utilized to assess $CD4^+$ T cell proliferation after treatment with the anionic TSY (MAGE-$3_{281-295}$ peptide; Table 1) MHP-KDO2 nanoliposome. Firstly, a cell viability study was performed using a live-dead marker and flow cytometry to ensure that cell viability was not impacted by the presence of either the peptides or the liposomes. In this study, SIN lymphocytes from the 2 patient donors (SIN1and SIN2) were treated with a variety of free peptides and liposome combinations. For the free peptide group, the treatments included: no peptide and GAG peptide as negative controls, a mixture of all 6MHPs, and the single TSY peptide of interest. For the liposome group, the cells were treated with ghost liposomes, liposomes containing KDO2 but no peptide, and TSY containing liposomes both with and without KDO2. A control cell line was used as a viability comparison. Next, in order to assess CD4 T cell proliferation, we split the SIN1 and SIN2 cell lines into five treatment groups. These were: free non-encapsulated TSY, a ghost nanoliposome, a ghost KDO2 nanoliposome, a TSY encapsulated (no KDO2) nanoliposome, and a combinatorial KDO2/TSY nanoliposome (full vaccine). A CFSE dye-dilution proliferation assay was performed to evaluate the donor immune response by flow cytometry. Specimens were thawed and labeled with carboxyfluorescein diacetate (CFSE; Vybrant® CFDA SE Cell Tracer Kit, Invitrogen™, ThermoFisher Scientific) according to the manufacturer's instruction, with a final dye concentration of 1 μM. Two hundred thousand labeled cells were added to flat bottom wells of a 96-well cluster plate (Falcon, ThermoFisher) containing peptides in solution or in nanoparticle form. The final peptide concentration was 2 μg/mL in a final culture volume of 0.2 mL. We also utilized a media-only and

an HIV GAG peptide as negative controls[40]. To avoid cell starvation from the high PBS content of the nanoliposomes' solvent relative to the necessary culture media nutrient content to sustain the culture during incubation, cells and liposome-containing formulations of peptide (and liposome controls) were first incubated ("pulsed") for 2 h at 37 °C followed by centrifugation to pellet the cells (and absorbed or internalized nanoparticles). The "pulsing" medium supernatant was removed and replaced with a complete culture medium consisting of AIM V (Gibco/Life Technologies, ThermoFisher) supplemented with 5% human AB serum (Gemini Bioproducts). Treatments were incubated for five days. At the end of incubation, cells were collected and labeled with a live/dead fixable dye (Aqua; ThermoFisher), followed by labeling with CD3 v450, CD4 PE, and CD8 PE-Cy7 (BDBiosciences). Cells were acquired on a Canto II flow cytometer (BDBiosciences) maintained by the Carter Immunology Center at the University of Virginia and the data were analyzed using FlowJo software (version 10; BDBiosciences).

RESULTS

Physico-chemical characterization of 6MHP nanoliposomal formulations

We initially incorporated each of the 6 melanoma helper peptides (6MHP) in a neutral liposome formulation containing: (DSPC); (DOPE); (PEG2000PE); KDO2; cholesterol; and a fluorophore in ratios of 4.60:2.14:0.25:0.01:3.00:0.02 respectively [Table 2]. After quantifying initial encapsulation masses, we utilized an iterative approach to improve encapsulation via modifying the charge and lipid ratios of the liposomal formulation as well as the buffer for the peptide. The proportional improvements in encapsulation, as well as the encapsulation efficiencies, are depicted in Table 3.

Using dynamic light scattering (DLS, Malvern Instruments), we demonstrated that all 6 optimized formulations displayed stable, consistent, and homogeneous size distribution, with an overall average size range of 113 ± 8 nm when averaged among all 6 formulations [Figure 1]. Furthermore, Figure 2 shows that all 6 formulations could be mixed together and remain stable in suspension, providing confidence that the charged nanoliposomes are not interacting with each other to cause destabilization or aggregation. The mixed sample gave a Z-average size of 113.5 nm, which is in agreement with our average size distribution of the individual nanoliposomes.

In addition, results of a 5-week long release kinetics study and analysis by LC-MS did not detect any measurable amount of peptides present in the MHP-KDO2-nanoliposome supernatant when stored at 4 °C (data not shown). These data suggest that the nanoliposomes do not have a significant release of encapsulated peptides from their core for at least that length of time, while under typical refrigerated storage conditions. Additionally, the supernatant collected remained clear, providing confidence that the fluorophore was not released from the liposome and DLS analyses still showed good liposome stability. Conversely, at body temperature, greater than 50% of the peptide concentration was released within the first 24 h. DLS data showed that the liposome remained intact, even in 10% bovine serum and the supernatant remained clear of the fluorophore. After one week, the liposomes kept at room and body temperature did appear to begin breaking down, shown by an increased number of peaks in the DLS analyses (data also not shown).

Murine biodistribution studies

A biodistribution study using a mixture of all 6 MHP formulations was performed using an IVIS Spectrum imaging system. Since each liposome formulation contained a different peptide mass, the mixture was prepared to contain equal amounts of each peptide (i.e., different volumes of each liposome solution to maintain equal peptide delivery). For this particular study, the nanoliposome vaccine was prepared using (DiD) fluorophore, as DiD has a far-infrared emission spectrum conducive to live animal imaging via IVIS.

Table 2. Finalized liposome formulations for the neutral, cationic and anionic nanoliposomes developed to encapsulate different peptides based on their residual charge. A dissolution buffer maintaining each peptide's optimal pH range was also used during fabrication, where B1= 1.33 mg/mL sodium bicarbonate ($NaHCO_3$) in a 1:2 solution of Lactated Ringers solution (LR) and water respectively; B2 = 5 mg/mL $NaHCO_3$ in water; B3 = 1 mg/mL $NaHCO_3$ in water; and B4 = 1:9 ratio of 2-(N-morpholino) methanesulfonic acid (MES) buffer and water

Lipid Component	Molar Ratio		
	Neutral (FFL,LLK,RNG)	Cationic (AQN)	Anionic (WNR, TSY)
DSPC	4.60	4.12	3.91
DOPE	2.14	1.90	1.81
PEG(2000)-PE	0.25	0.25	0.25
KDO2	0.01	0.01	0.01
Cholesterol	3.00	3.00	3.00
Rhodamine (or DiD)	0.02	0.02	0.02
DOTAP	-	0.70	-
DHP	-	-	1.00
Buffer	B1	B2	B3 (WNR), B4 (TSY)

DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine; DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt).

Table 3. Showing both the Initial peptide encapsulation mass within our neutral nanoliposome formulation when the peptides are dissolved in 1 × PBS; compared to the encapsulation efficiencies within our optimized nanoliposome formulations, where each peptide is also dissolved in an aqueous solution that maintains optimal pH, peptide stability, and dissolution. Encapsulation efficiency is based upon initial 500 μg/ml peptide concentration. All peptides were calculated from a full vaccine, which included all lipid components (i.e. adjuvents, PEG, peptides). Each optimized mass encapsulation is based on *n* = 3 separate experiments, repeated in triplicate

Peptide	Original mass encapsulation using neutral formulation and 1X PBS (μg/mL)	New liposome formulation (Charge and buffer)	pH range	Optimized mass encapsulation (μg/mL)	Encapsulation improvement	Encapsulation efficiency (%)
AQN	0.02 0.005	Cationic (B2)	8.5-9.0	6.25 1.13	312X	1.25
WNR	4.81 0.09	Anionic (B3)	7.0-8.0	7.64 0.99	1.59X	1.53
LLK	34.56 0.69	Neutral (B1)	6.0-8.0	57.26 14.39	1.66X	11.45
FLL	28.15 2.05	Neutral (B1)	6.0-8.0	58.61 4.96	2.08X	11.72
RNG	22.37 0.30	Neutral (B1)	6.0-8.0	19.18 6.36	0.85X	3.29
TSY	0.17 0.001	Anionic (B4)	5.0-5.5	141.35 2.18	831X	28.27

AQN: AQNILLSNAPLGPQFP; WNR: WNRQLYPEWTEAQRLD; LLK: LLKYRAREPVTKAE; FLL: FLLHHAFVDSIFEQWLQRHRP; RNG: RNGYRALMDKSLHVGTQCALTRR; TSY: TSYVKVLHHMVKISG.

We compared routes of administration, SQ vs. IV. The IVIS images on day zero (immediately after injection) and day 6 (6 days after injection) are shown in Figure 3. The mice injected SQ displayed little biodistribution on day zero [Figure 3A], which intensified within a region consistent with liver and spleen on day 6 [Figure 3C]. In contrast, images of the mice injected IV with nanoliposome vaccines show rapid, systemic biodistribution in both mice on day zero [Figure 3B], but the tissue fluorescence diminished by day 6 [Figure 3D] as compared to the fluorescence on day zero. On day 6, the mice were sacrificed and their organs were harvested. Quantitative fluorescence measurements were performed using Aura region of interest (ROI) programming and software (see methods), and these results are shown in Figure 4.

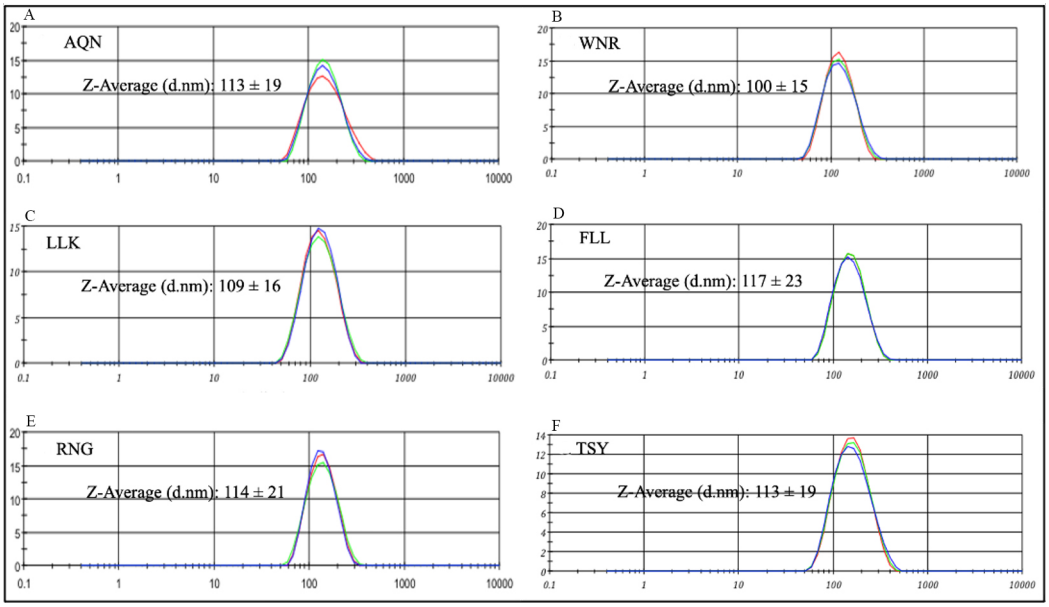

Figure 1. Typical dynamic light scattering (DLS) data attained for each of the 6 melanoma helper peptides. The data shows size distribution by intensity with size, d in nanometers along the X-axis and percentage intensity on the Y axis. (A) shows the DLS profile of the AQN peptide; (B) the DLS profile of the WNR peptide; (C) is that of LLK; (D) FLL; (E) RNG; and (F) is the typical profile for the TSY peptide. The graphs show a homogenous size distribution for all 6 peptides. The Z-average size (d.nm) attained from multiple samples is also provided for each peptide. AQN: AQNILLSNAPLGPQFP; WNR: WNRQLYPEWTEAQRLD; LLK: LLKYRAREPVTKAE; FLL: FLLHHAFVDSIFEQWLQRHRP; RNG: RNGYRALMDKSLHVGTQCALTRR; TSY: TSYVKVLHHMVKISG.

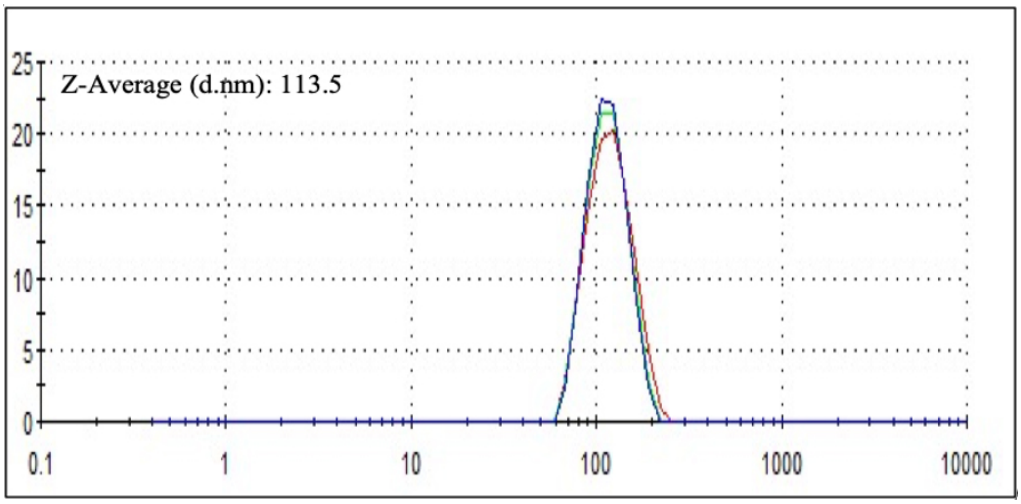

Figure 2. Dynamic light scattering data attained after mixing all 6 peptide nanoliposome formulations together. The data shows size distribution by intensity, with size (d) in nanometers along the X-axis and percentage intensity on the Y axis. The graphs show a homogenous size distribution. The Z-average size (d.nm) attained for this data is 113.5 nm.

For all organs, the mice injected SQ displayed a higher concentration of fluorescent liposomes within the harvested organs than the IV-injected group. No overt signs of toxicity (lethargy, loss of appetite, weight loss) were observed in any of the mice over the study. Taken together, these data suggest that both IV and SQ administration of a KDO2-6MHP nanoliposome is safe, and that SQ administration of the KDO2-6MHP nanoliposome offers a robust delivery of cargo to critical secondary lymphoid organs (spleen and lymph nodes), which persists at least 6 days.

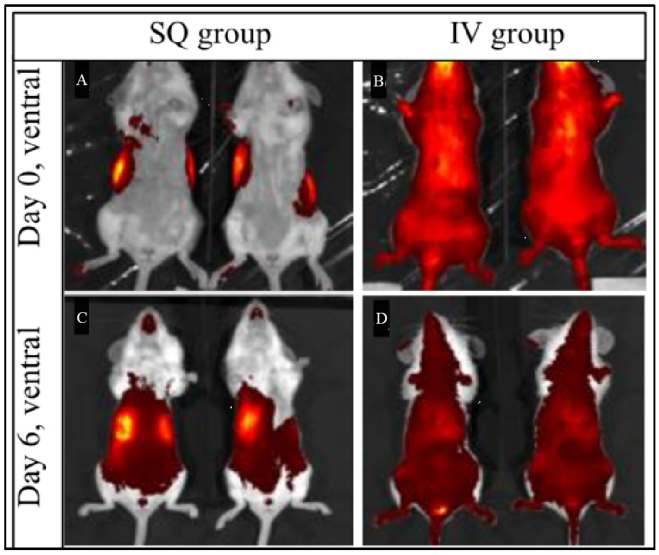

Figure 3. Fluorescent imaging of liposome biodistribution in mice on days 0 and 6. Two mice (SQ group) were injected subcutaneously in either flank and 2 mice (IV group) were injected intravenously.

urinActivation of CD4⁺ T cells from patients by a KDO2-MHP nanoliposome

Using blood samples from melanoma patients who had previously been vaccinated with a 6MHP vaccine on the Mel41 trial and had developed a robust CD4⁺ T cell immune response to the TSY antigen[39], we analyzed lymphocyte viability and CD4⁺ T cell proliferation to the TSY nano-formulations *ex vivo*. Under IRB guidance, we had previously collected fresh lymphocytes and sentinel immunized nodes (SIN) from these patients during that prior clinical trial. SIN were nodes draining the vaccine site, identified by lymphatic mapping techniques[39,41] Using a live-dead marker and flow cytometry, we observed no discernable differences in cell viability between each of the patient samples re-treated with free non-encapsulated TSY peptide and the nanoliposomal KDO2/TSY formulation; however, the SIN sample from patient 2 (SIN2) had a decreased viability compared to SIN patient 1 (SIN1, Figure 5A). Viability was equivalent between a control group of patient samples that were not previously immunized with peptides [Figure 5A]. Since there was no apparent toxicity to incubation with nanoliposomes, we treated aliquots of each cell preparation with each of the following: (1) an empty ghost liposome; (2) a liposome containing KDO2 but no peptide; (3) Free TSY peptide (no liposome); and (4) TSY encapsulated within a liposome; and v) TSY within an immunogenic (KDO2 containing) liposome. Then the average proliferation of CD4⁺ gated cell populations for various culture treatment conditions was assessed [Figure 5B] by CFSE dilution over five days. Cell cultures were expanded from the harvested SIN biopsy of each donor, and CD4 T cell proliferation was assessed by CFSE dye dilution. Proliferation is reported as the percentage of CD3⁺ CD4⁺ gated population that have dividing (CFSE-diluted). Compared to all controls, both SIN1 and SIN2 donor cells responded to the combinational therapy. Figure 5C shows the 2D histograms of the same data against a negative control. Peripheral blood samples from each patient had no significant response to any of the treatments (data not shown). While an expected CD4⁺ T cell proliferation response is observed with free TSY and liposomal TSY, our data suggest an advantage of combining the peptide vaccine with a TLR4 agonist. It is worth noting that neither the liposome alone nor the KDO2-containing liposome alone triggers CD4⁺ proliferation, and thus KDO2 only acts to enhance the response to the peptide of interest. It should also be noted that while the SIN2 patient response is weaker than SIN1 [Figure 5B], this can be attributed to the slightly lower viability [Figure 5A] (and hence fewer live cells present) in the SIN2 donor cell population. Taken together, we demonstrate an immunogenic response to the KDO2/MHP nanoliposome *ex vivo*.

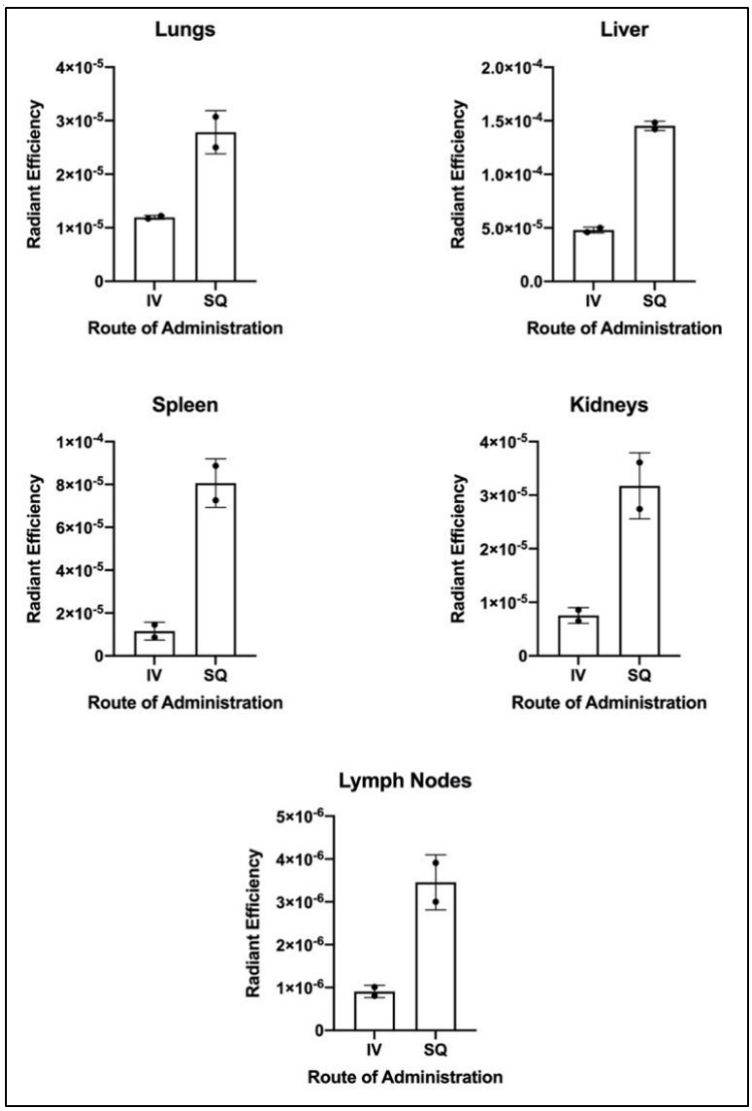

Figure 4. Showing the mean radiant efficiency in the extracted organs of four mice, 2 of which were injected with fluorescent and immunogenic liposomes via intravenous (IV) injection and 2, who were administered the same nanoliposomes via subcutaneous (SQ) injection in either flank.

DISCUSSION

In this study, we have developed nanoliposome formulations to encapsulate a specific panel of 6 melanoma helper peptides as a first step toward clinical application as a new melanoma vaccine strategy. The 6 peptides vary in length, hydrophobicity/hydrophilicity, and isoelectric point, which required creating 3 different nanoliposomal formulations to encompass them. These 6 peptides have previously shown modest immunogenicity and clinical activity as a vaccine when injected in their free form with other immunogenic adjuvants. Our goal was to improve future clinical efficacy through nano-enhancement strategies via simultaneous delivery of all 6MHPs and corresponding immunogenic adjuvants in a single nanoliposomal solution. In these investigations, we incorporated KDO2, a TLR4 agonist, into the lipid bilayer as our immune-stimulating adjuvant. Our results demonstrate that the individual liposomal formulations remain stable after being mixed together and that they can be simultaneously delivered without apparent toxicity *in vivo*. We show that via SQ injection, these 6MHP-loaded liposomes are capable of diffusing rapidly to

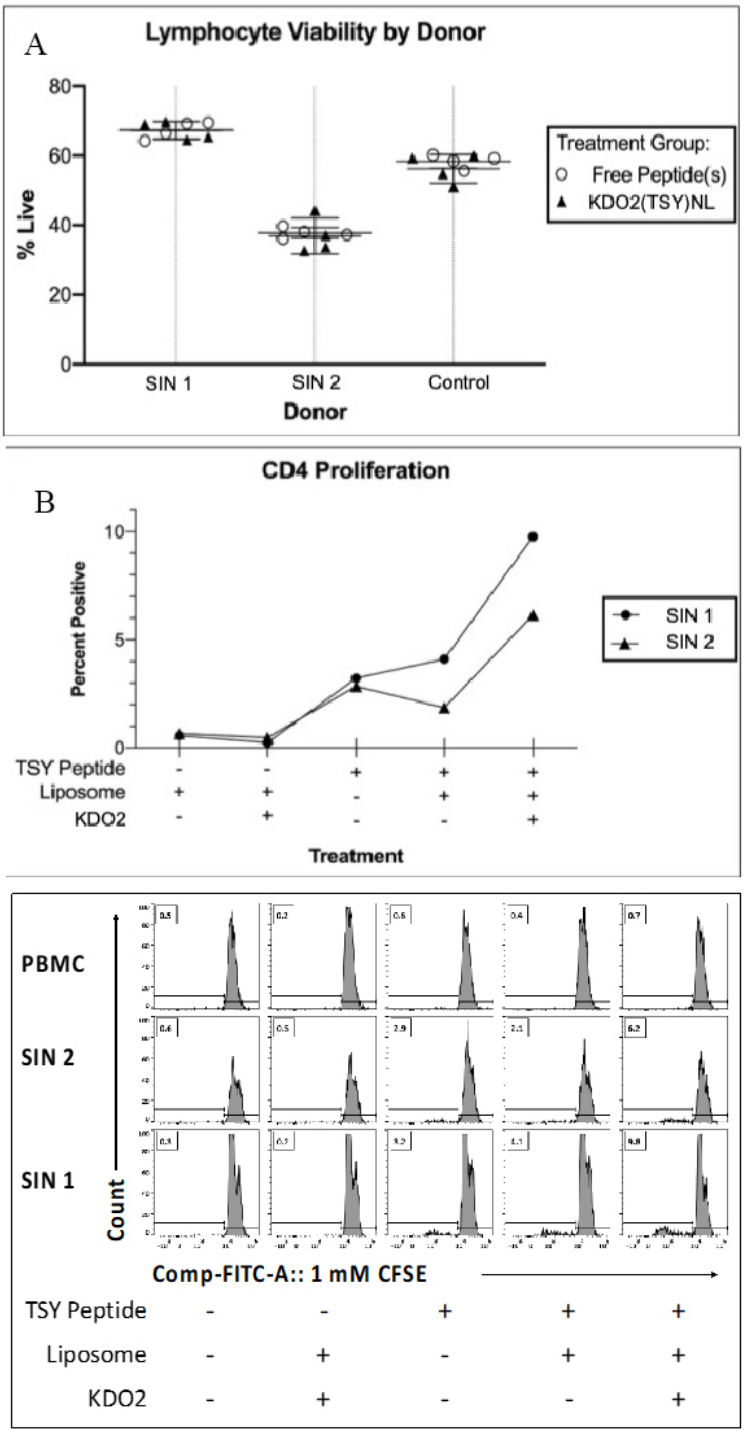

Figure 5. (A) Graph of lymphocyte viabilities of Sentinel Immunized Nodes (SIN) post-treatment. Thick horizontal bars represent mean viability among treatments for a donor; (B) showing a graph of average proliferation of $CD4^+$ cell populations after culture treatments with (i) an empty anionic liposome, (ii) an anionic liposome containing KDO2 but no peptide; (iii) Free TSY (no liposome); (iv) TSY encapsulated in an anionic liposome; and (v) an anionic liposome containing TSY and KDO2; and (C) showing the 2D histograms used to calculate the data in 5B. PBMC: Peripheral blood mononuclear cells.

secondary lymphoid organs and appear to remain in circulation for at least 6 days. We also show that these immunogenic liposome formulations significantly enhance immune responses to specific peptides *ex vivo*.

The initial development of our nano-enhanced vaccine was complicated, as we were required to encapsulate and ultimately deliver 6 different peptides simultaneously. We selected a nanoliposome framework because of its versatility. Nanoliposomes can be made with a variety of different lipids to construct the lipid bilayer. They can be made to be neutral or charged, and the exterior surface can be modified chemically for the addition of targeting ligands that enhance liposome delivery; and/or stealth properties that increase biocompatibility and circulation time in the body[42-44]. Hydrophilic compounds are typically encapsulated within a nanoliposome's aqueous interior, while hydrophobic (or lipophilic) compounds are typically embedded within the lipid bilayer. However, the development and optimization of nanoliposomes is not always a straightforward process, as overall charge, distribution of charge, and size, as well as ionic buffer strength, affect encapsulation efficiency. Each of our peptides had different physical properties, so a single liposome formulation was not optimal for this range of peptides. Instead, we engineered 3 formulations and encapsulated each peptide based upon pH-dependent solubility. While each formulation may have a different surface charge or be formulated with a different buffer system, they still all include a fixed PC/PE ratio that maintains stability, a fixed cholesterol content that prevents leakiness, a fluoroprobe to facilitate *in vivo* imaging, a reduced PEG brush to help trigger the body's T cell response to these liposomes, and a low concentration of the TLR4-agonist, KDO2, to enhance the adaptive immune response without inducing systemic toxicities. Figure 6A shows a schematic diagram of our base nanoliposome formulation, and Figure 6B shows the chemical structure of KDO2-Lipid A.

The new formulations and pH-controlled buffers improved the encapsulation efficiency of most of the peptides compared to the use of a generic neutral nanoliposome formulation. TSY was improved the most. TSY is most stable in pH 5-5.5; thus, PBS did not provide the ideal buffer conditions, while the MES buffer used in our optimized formulations was much more favorable. The anionic liposome formulation also aided in improving the encapsulation of the positively-charged TSY peptide. Similarly, AQN, which had very poor encapsulation in our neutral formulation, also achieved huge encapsulation enhancements when dissolved in a buffer that maintained an alkaline pH and by using a cationic charged nanoliposome to further enhance the encapsulation of the negatively charged peptide. By contrast, encapsulation of WNR, which has a slightly positive charge, was only marginally improved after switching to an anionic liposome and buffer that maintained a pH of 7.0-8.0. LLK, FLL, and RNG achieved reasonable encapsulation values in our neutral formulation, so we did not expect significant improvements with a switch of buffers. However, for LLK and FLL, but not RNG, dissolving the peptides in LR/$NaHCO_3$ buffer, instead of PBS, slightly improved encapsulation efficiencies. Based on previous clinical studies, where 200 ug of each peptide was delivered to human patients[39], we estimate that a liposomal loading value of ~50ug/ml should yield an effective immunologic dose. Our studies show that LLK, FLL, and TSY can be reengineered to reach and/or exceed this therapeutic dose. For AQN, WNR, and RNG, we may also attempt pH-dependent active methods of liposomal loading to further enhance encapsulation of these peptides within these nanoformulations. However, based upon previous studies from our group[22], we might expect that the nanoformulations, in fact, better protect and deliver the peptides, allowing suboptimal encapsulation efficiencies to now reach target therapeutic doses.

In future studies, to further improve liposomal MHP encapsulation, we may also attempt to modify the peptides via techniques such as myristoylation[45] or palmitoylation[46]. Both of these methods have been shown to enhance peptide-lipid interactions, allowing for intercalation of the peptides within the lipid bilayer. We chose not to adopt this process initially for several reasons. First, as we already intercalated cholesterol, KDO2, and our fluorophore within the lipid bilayer, we wanted our peptides to sit within the aqueous core of the liposomes in their native form. We were also concerned that incorporation within the lipid bilayer might lower the efficacy of our therapy due to prolonged release kinetics and/or destructive

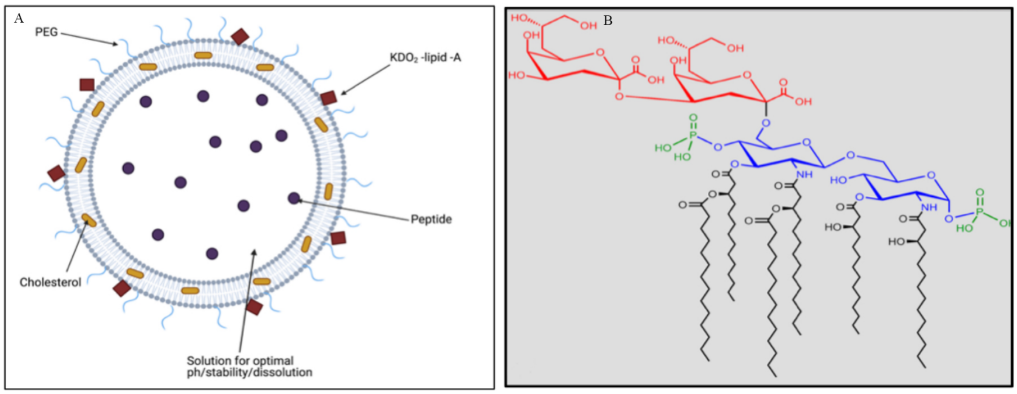

Figure 6. Showing (A) a schematic diagram of the unilamellar nanoliposome with the peptide dissolved within a stabilizing pH controlled buffer within the aqueous liposome core. Cholesterol embeds within the lipid bilayer; and a sparse PEG brush and immunogenic KDO2 lipid head group are arranged around the outer shell of the nanoliposome. The liposome is stored as a suspension in 1X PBS. It should be noted that additional lipid components are added to create either a positive or negative charge in some of the formulations. A fluorophore may also be added, which depending on the fluorophore used, may embed within the bilayer like cholesterol or be attached to a lipid and incorporate within the bilayer like PEG and KDO2. (B) shows the structure of KDO2-lipid A, which has 6 fatty acid chains and a head group on the surface.

interaction/competition with the other adjuvants already in the lipid bilayer. We were also uncertain about how such modifications (and changes in peptide configuration) might affect the immunogenicity of the parent MHP. As part of this ongoing work, we plan to test whether these (and similar) modifications preserve or alter the immunogenicity of the 6MHPs.

A major innovation of our nano-encapsulated approach is the incorporation of the immunogenic KDO2-lipid A into our core nano-liposome formulation. The present study demonstrates the feasibility of creating customized nanoliposomes, containing varying peptides, which can then be mixed for storage and co-administration. These data provide a platform on which to build even more promising nanoliposome strategies. One we propose is to add an antibody that can target the adjuvanted nanoliposomes specifically to dendritic cells. In murine studies, very strong circulating T cell responses, representing about 50% of circulating T cells, have been induced by IV vaccination with peptides plus a TLR agonist and a CD40 antibody that targets antigen-presenting cells (TriVax)[47-50]. However, comparable systemic dosing of those agents for humans is not likely achievable without unacceptable toxicity. If, on the other hand, these 3 agents could be co-administered in a nanoparticle targeted to dendritic cells (DC), it may prove as effective with a much lower dose. Future versions of our immunogenic liposomes will include bioconjugation strategies (EDC/NHS) for binding CD40 Ab to the surface of the liposomes.

Our biodistribution studies, conducted in healthy murine subjects, suggest that SQ administration could be a preferred method of delivery for our nano-vaccines. Systemic (IV) delivery seems to rapidly distribute the liposomes non-specifically throughout the body immediately after injection, and also appears to then produce a more rapid clearing of them from the body. On the other hand, SQ delivery allows for a slower and more controlled distribution. We acknowledge that a limitation of this present work includes not knowing precisely the extent to which the fluorescent tracer may be released over time *in vivo*. However, as the fluorophore is conjugated to a lipid within the bilayer, we do not expect significant amounts of tracer to be released until the liposomes themselves begin breaking down. Our benchtop studies at body temperature in 10% bovine serum showed that while we observed peptide release after 24 h, the liposomes themselves remained stable for several days. We are confident that the fluorescent distributions imaged immediately after injection on day zero [Figure 3A and C] are representative of liposome distribution at that time. Unlike

IV delivery, SQ delivery does not immediately distribute systemically but does show the rapid appearance of the liposomes within the lymph nodes and spleen. This is encouraging and implies that the nanoliposomes exhibit immunogenicity. It also suggests that even without specific dendritic cell targeting, the nanoliposomes distribute in tissues with high concentrations of dendritic cells, which can be expected to support T cell activation. In future studies, we intend to conduct more detailed benchtop tests to specifically monitor fluoroprobe release over time, as well as increase the frequency of live animal imaging. We also plan to extend this work into a B16 melanoma mouse model. This will require exchanging our humanized antigens with mouse antigens, which may require some alterations to the nanoformulations and thus was beyond the scope of this manuscript. However, this kind of study will allow us to evaluate biodistribution, pharmacokinetics (PK), and immunogenicity of the KDO2-nanoliposomes, as well as determination of pharmacodynamics (PD) for the efficacy of our nanoformulations in a mouse model. We would also like to repeat studies in mice with depletion of dendritic cells to confirm the reliance on those cells.

The goal of the present work was to test whether the 6MHPs could be encapsulated into nanoliposomes with a TLR agonist and whether these would be stable and show preliminary evidence of enhanced immunogenicity. In our preliminary investigations, the TSY peptide consistently achieved the highest liposomal encapsulation values and was also strongly immunogenic in a melanoma patient population[41]. Our team also had access to 2 patient samples, previously documented to exhibit a strong TSY immunogenic response. Thus, as a proof-of-concept, we evaluated proliferative responses of human $CD4^+$ T cells to the TSY peptide *ex vivo* using these 2 patient samples. Our assays showed an enhanced immunogenic response with the peptide encapsulated within a KDO2-nanoliposome, compared to formulations without KDO2 and the free peptide. We expect that this enhancement is mediated by dendritic cell activation and subsequent helper peptide presentations to melanoma-reactive T cells. These results are in line with clinical data demonstrating that simultaneous delivery of a TLR4 agonist with immunogenic peptides enhances T-cell activation[16]. These initial studies provide a basis for carrying forward this study, first by performing *ex vivo* activation of the five remaining antigens individually, as well as *ex vivo* analysis of the "full vaccine" with a mixture of all 6 peptide nanoliposomes.

Future experiments to support the preclinical development of our nano-formulations include formal PK and toxicological studies by a contract research organization, in addition to the determination of PD for efficacy in a mouse model.

Our results show promise in the use of custom-designed immunogenic (KDO2) nanoliposomes as the delivery vehicle for cancer vaccines. Co-delivery of antigens plus the TLR agonist KDO2 to antigen-presenting cells offer promise to enhance immune responses to melanoma antigens. Prior vaccines in humans commonly induce weak or transient T cell responses: by enhancing those immune responses, this new strategy offers promise to overcome weak antitumor immunity and enable immune-mediated control of melanoma. Moreover, by improving the delivery and efficacy of nano-cancer vaccines, drug resistance to current chemotherapies can also be overcome. Our mouse studies show that a subcutaneous injection may have advantages over IV injection, as it allows the nanoliposome vaccine to concentrate and persist within tissues and organs with high DC populations. This will provide an easier route to immunization that is cost-effective and less invasive for the patients receiving care. Co-administering diverse adjuvants within a nanoliposome are expected to show even further enhanced responses *in vivo*, as it provides a way of targeted delivery, ensuring that all adjuvants are delivered directly and simultaneously to DCs.

DECLARATIONS

Acknowledgements

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Authors' contributions

Prepared and optimized nanoliposome formulations; nano-characterization analysis; *in vivo* Murine studies; preparation of data; writing: Salotto KE
Conducted *ex vivo* cell studies; analysis and preparation of data: Olson Jr WC.
Developed *in vivo* murine study protocol: Pollack KE
Preparation and characterization of nano-liposomes: Illendula A
Developed early-stage nano-liposome formulations: Michel E
Preparation and characterization of nano-liposomes; writing: Henriques S
Mass Spectrometry analysis: Fox T
Mass Spectrometry analysis: Walker S
Directed *ex vivo* studies; writing; editing: Slingluff Jr CL
Adviser for nano-liposome studies; writing; editing: Kester M
Designed and Directed nano-liposome formulation development and analysis; writing; editing: Snyder HW
Prepare and conduct the murine imaging studies: Dunlap-Brown M

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study included murine *in vivo* studies and experiments using human lymphocytes. The murine studies were approved by the University of Virginia Animal Care and Use Committee (ACUC), as Protocol number 4255. The lymphocytes were isolated from lymph nodes excised as part of a prior clinical trial (Mel41; NCT00089219), which was approved by the University of Virginia Institutional Review Board for Health Sciences Research under IRB #10464, and by the U.S. Food and Drug Administration under Initial New Drug (IND) application #10825. All human participants signed written informed consent for use of their samples for analysis of immune responses. Participants were assured that they had the right to leave the study at their convenience. Patients' samples were identified through a code number (VMM#). Clinicopathological data of patients were confidentially kept, and the electronic information was stored on a password-protected central server at the University of Virginia.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Editorial

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New insights for drug resistance in metastatic castration-resistant prostate cancer

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Abstract

Prostate cancer is the most common cancer and is the second leading cause of cancer-related deaths among men in the United States. Androgen deprivation therapy (ADT) is the standard treatment for advanced-stage prostate cancer; however, this treatment eventually fails, leading to an incurable disease subtype known as metastatic castration-resistant prostate cancer (mCRPC). There are several molecular mechanisms that facilitate the development of mCRPC engaging androgen receptor (AR) growth axis, including AR amplification, gain of function AR mutations, and AR splice variants that are constitutively active and are a foremost factor for mCRPC development. AR-independent mechanisms with exceptionally low or absent AR expression found in cancer cells suppress ADT effectiveness and contribute to aggressive variants, including neuroendocrine differentiation. Several other AR regulatory factors such as epigenetic modification(s), and DNA damage response have been reported during post-ADT exposure and play a crucial role in mCRPC development. Therefore, targeting prostate cancer cells before their progression to mCRPC would improve patient outcomes. This special issue in "Cancer Drug Resistance" focuses on understanding the mechanism(s) and development of mCRPC resistance. This special issue also highlights the therapeutic strategies to combat against resistant subtype. This issue comprehensively reviews the mCRPC and delivers the update in the forum of mCRPC resistance development.

Keywords: Castration-resistant prostate cancer, drug resistance, cancer stem cells, androgen receptor, neuroendocrine differentiation, extracellular vesicles, chemotherapy, androgen receptor

INTRODUCTION

In recent years, studies related to metastatic castration-resistant prostate cancer (mCRPC) have received global attention. mCRPC is an aggressive and advanced form of prostate cancer, having progressed to various organ sites and no longer responding to androgen deprivation therapies that result in lowering of testosterone[1-2]. Although several treatment options are available for mCRPC patients, the disease remains incurable, with a poor prognosis and low survival rates[3]. Current systemic treatment options for mCRPC include hormonal therapy, chemotherapy, immunotherapy, radiotherapy, and bone-modifying agents[4]. There are few recently included novel therapies such as PARP inhibitors and immune checkpoint inhibitors including PD-1/PD-L1 and CTLA4 for a subset of mCRPC patients[5]. The special issue of "Drug resistance in metastatic castration-resistant prostate cancer" provides insights into the targets and treatment of mCRPC.

ARTICLE DESCRIPTION

In the present special issue, a total of six articles have been qualified and published. The first review published by Yehya *et al.* (2022) shed the light on emerging targeted therapies currently evaluated in clinical trials with promising potential to overcome mCRPC-drug resistance[6]. This review also provides the updated mechanism of action of mCRPC development. The authors highlight the role of cytochrome P450 enzyme 17 (CYP17) in steroidal biosynthesis, especially androgen synthesis. Implications of altered cellular signaling pathways, kinases, and other factors such as cytokine and enzymes that endorse the mCRPC resistance are described in this review. The authors also highlight the completed and ongoing clinical trials against mCRPC, including PARP inhibitors, immunotherapies, AR degraders, and PI3K signaling inhibitors.

Another original article by Kumar *et al.* (2022) showed the role of extracellular vesicles secretion in paclitaxel resistance of prostate cancer cells[7]. Treatment of paclitaxel (PTX) resistant prostate cancer cells with GW4869 significantly reduced the release of small EVs (50-100 nm size range) while increasing the release of larger EVs (> 150 nm in size) and inhibited their clonogenicity. Moreover, GW4869 treatment significantly inhibited the survival of PTX-resistant prostate cancer cells in a dose-dependent manner and reduced the tumor weight in an *in vivo* model.

Kushwaha *et al.* (2022) performed a critical review and demonstrated the role of prostate cancer stem-like cells (PCSCs) in the development of antiandrogen resistance[8]. PCSCs have the ability to differentiate, self-renew, and regenerate tumor heterogeneity. A variety of factors contribute to increasing PCSCs stemness including AR variants, AR mutation, and epigenetic and genetic alterations that lead to changes in the tumor microenvironment, such as adenosine 5′-triphosphate (ATP)-binding cassette (ABC) transporters expression and molecular signaling pathways. This review explored the molecular pathways in the generation of prostate cancer stem-like cells post ADT, various PCSCs markers, and a list of genes, their type and location along with fold change values associated with hematopoiesis pluripotent stem cells. The methods for isolation, identification, and characterization of PCSCs from primary prostate tumors have also been described.

Choi *et al.* (2022) provided a perspective on the transcription factor ONECUT2 which mediates AR-independent cell growth and neuroendocrine differentiation in CRPC[9]. It is a key factor in NE differentiation between adenocarcinoma to neuroendocrine prostate cancer (NEPC). The perspective specified genes including AR regulatory genes, FOXA1/2, and hepatocyte nuclear factor (Hnf) are the target(s) of ONECUT1 and ONECUT2. Specifically, ONECUT2 regulates the signaling of hypoxia-inducing factors and consequently promotes prostate cancer differentiation and can also serve as a therapeutic target in mCRPC.

Khalil *et al.* (2022) summarize the multifaceted role of Tousled-like kinase 1 (TLK1) in mCRPC progression and development of therapeutic resistance[10]. The highlight of this review includes that DDR kinase TLK1 mediates an important aspect of adaptation to androgen deprivation and promotes cell cycle progression under unfavorable growth conditions such as ADT and reprograms prostate cancer cells to adapt to androgen-independent growth and resistance development.

The final review article by Biersack *et al.* (2022) demonstrated that epigenetic modification of histone deacetylase (HDAC) plays a significant role in different mechanisms contributing to the development and persistence of CRPC chemoresistance[11]. The clinical trials on HDAC inhibitors for the treatment of CRPC were reviewed and the major outcomes have been summarized. Several other new HDAC inhibitors and the effect of HDAC inhibitors in combination therapies using anticancer drugs were highlighted.

Altogether, this special issue is a comprehensive review of the mechanism(s) of drug resistance and additional therapeutic opportunities for mCRPC treatment.

DECLARATIONS

Authors' contributions

Contributed to the conception and writing of the manuscript: Kushwaha PP, Gupta S

Conflicts of interest

Both authors declared that there are no conflicts of interest.

Availability of data and materials

Not applicable.

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Ethical approval and consent to participate

Not applicable.

Consent for publication

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Cancer Drug Resistance

Review

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Drug and apoptosis resistance in cancer stem cells: a puzzle with many pieces

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Abstract

Resistance to anticancer agents and apoptosis results in cancer relapse and is associated with cancer mortality. Substantial data have provided convincing evidence establishing that human cancers emerge from cancer stem cells (CSCs), which display self-renewal and are resistant to anticancer drugs, radiation, and apoptosis, and express enhanced epithelial to mesenchymal progression. CSCs represent a heterogeneous tumor cell population and lack specific cellular targets, which makes it a great challenge to target and eradicate them. Similarly, their close relationship with the tumor microenvironment creates greater complexity in developing novel treatment strategies targeting CSCs. Several mechanisms participate in the drug and apoptosis resistance phenotype in CSCs in various cancers. These include enhanced expression of ATP-binding cassette membrane transporters, activation of various cytoprotective and survival signaling pathways, dysregulation of stemness signaling pathways, aberrant DNA repair mechanisms, increased quiescence, autophagy, increased immune evasion, deficiency of mitochondrial-mediated apoptosis, upregulation of anti-apoptotic proteins including c-FLIP [cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein], Bcl-2 family members, inhibitors of apoptosis proteins, and PI3K/AKT signaling. Studying such mechanisms not only provides mechanistic insights into these cells that are unresponsive to drugs, but may lead to the development of targeted and effective therapeutics to eradicate CSCs. Several studies have identified promising strategies to target CSCs. These emerging strategies may help target CSC-associated drug resistance and metastasis in clinical settings. This article will review the CSCs drug and apoptosis resistance mechanisms and how to target CSCs.

Keywords: Cancer stem cells (CSCs), apoptosis, drug resistance, death receptor pathways, anti-apoptotic proteins, Bcl-2 family, c-FLIP

INTRODUCTION

The cancer stem cell (CSC) paradigm emerged from investigating a subpopulation of less-differentiated CD34+/CD38- cells possessing stem cell-like renewal ability and robust malignant-initiating capacity in acute myeloid leukemia (AML)[1]. Cancer cells from various types of cancers with these characteristics have since been identified in nearly all solid tumors, including cancers of the brain, breast, colon, pancreas, prostate, liver, lung, ovary, head and neck, stomach, thyroid, and melanomas[2]. The biological importance of activation targets of Nanog, Oct4, SOX-2, and c-Myc in CSCs, which are more frequently overexpressed in poorly differentiated tumors than in well-differentiated tumors, has been shown by correlating signature characteristics of these cells and poor survival[3]. Interestingly, specific dysregulated signaling pathways maintain CSCs renewal capacity with unique patterns among various tumor types. For instance, CSC maintenance in glioblastoma, colon cancer, gastric cancer, and prostate cancer is regulated by CD133-mediated AKT, leucine-rich G-protein-coupled receptor 5 (LGR5)-mediated Wnt/β-catenin and speckle-type POZ protein (SPOP)-mediated Nanog pathways[4-8]. Moreover, Wnt signaling cascades cross-talk with the FGF, Notch, Hedgehog (Hg), and TGFβ/BMP signaling pathways and regulate the expression of CSC markers, such as CD44, CD133 (PROM1), EPCAM, and LGR5 (GPR49) in these tumors[9]. In contrast, regulation of breast cancer CSCs (BCSCs) occurs by CD44 standard splice isoform (CD44s)-activated platelet-derived growth factor receptor b (PDGFRb)/signal transducer and activator of transcription 3 (STAT3), forkhead box C1 (FOXC1)-activated sonic hedgehog (SHH), and sphingosine-1-phosphate (S1P)/S1PR3-activated NOTCH pathways[10-13]. Therefore, these specific patterns of stemness regulation in various cancers have created significant complexity and specificity in various tumor types, which, in turn, may create a complicated situation with respect to therapeutic interventions aimed at eradicating CSCs from different tumor types.

Substantial data have provided evidence that tumors contain heterogeneous clones of CSCs and these cells are essential for tumor growth and survival[14]. Based on the CSC model, tumor heterogeneity due to clonal evolution of CSCs[15,16] is defined as cells with self-renewal capacity which are able to generate a progeny cell population [Figure 1]. As a result, the bulk of the tumor mass is differentiated and expanded progeny capable of rapid proliferation potential and harboring minor populations of various CSCs with particular properties, including their drug resistance phenotype [Figure 2]. Therefore, the major obstacle to curing tumors remains the presence of heterogeneous CSC clones resistant to chemotherapy and apoptosis[17-22]. Previous reports have proposed that targeting CSC subpopulations may result in tumor eradication and inhibition of tumor relapse[9,17-19]. However, tumors are curable when the heterogeneous CSC populations, as well as the rest of the tumor mass, including the progenitor cells and differentiated malignant cells, are targeted and eliminated[9,18,19].

The progression and heterogeneity of tumor cell populations may be explained by the CSC or cancer-initiating cell model[14-18] or by the clonal evolutionary model[14]. The CSC model, which is also referred to as the hierarchical model, states that tumors arise from a small percentage of CSCs that are derived from normal stem cells (NSC) that generate the bulk of tumor cell population[14,15] [Figure 2]. In the clonal evolution model, genetic and epigenetic changes happen over time in individual cells, and these alterations persist and provide a selective advantage; the clonal CSCs will outgrow other clones and result in a heterogeneous tumor population[14-18]. Interestingly, in the clonal evolutionary model, each cancer cell within the tumor is endowed with the potential to generate tumors having various degrees of drug-resistant

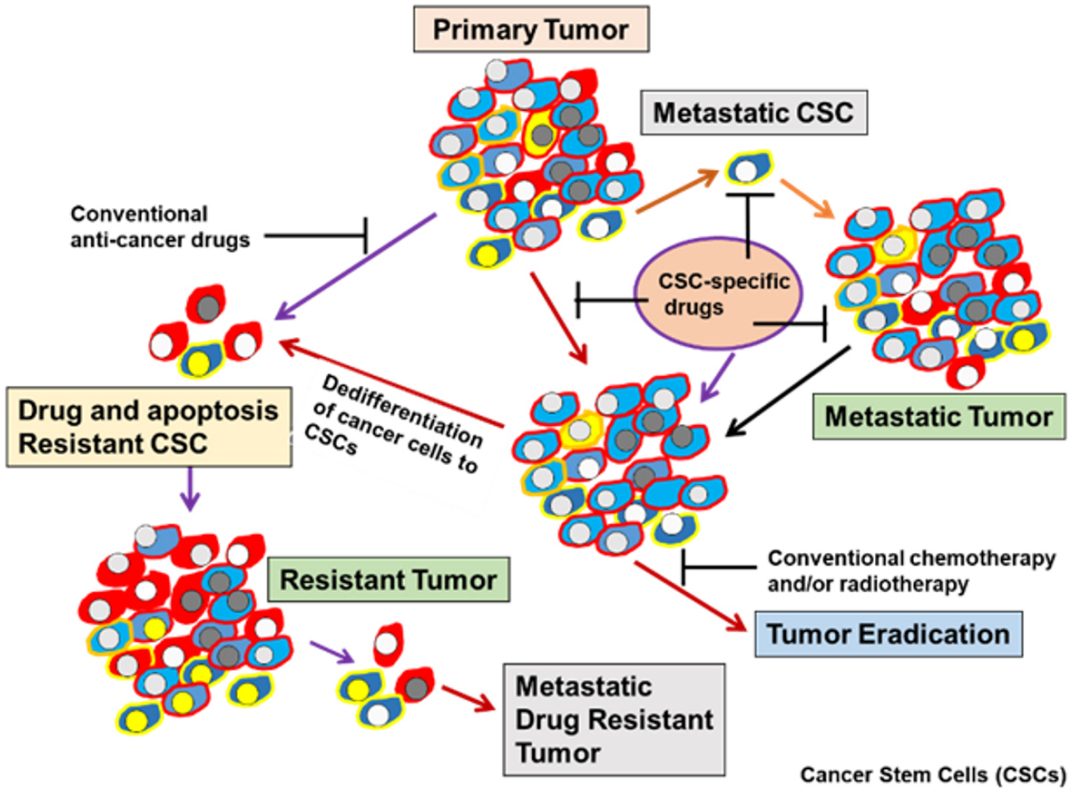

Figure 1. Heterogeneity of CSCs in tumors. Development of drug-resistance phenotype, metastatic tumor formation, and a potential strategy for eradicating tumors using CSC-specific drugs. CSC: Cancer stem cell.

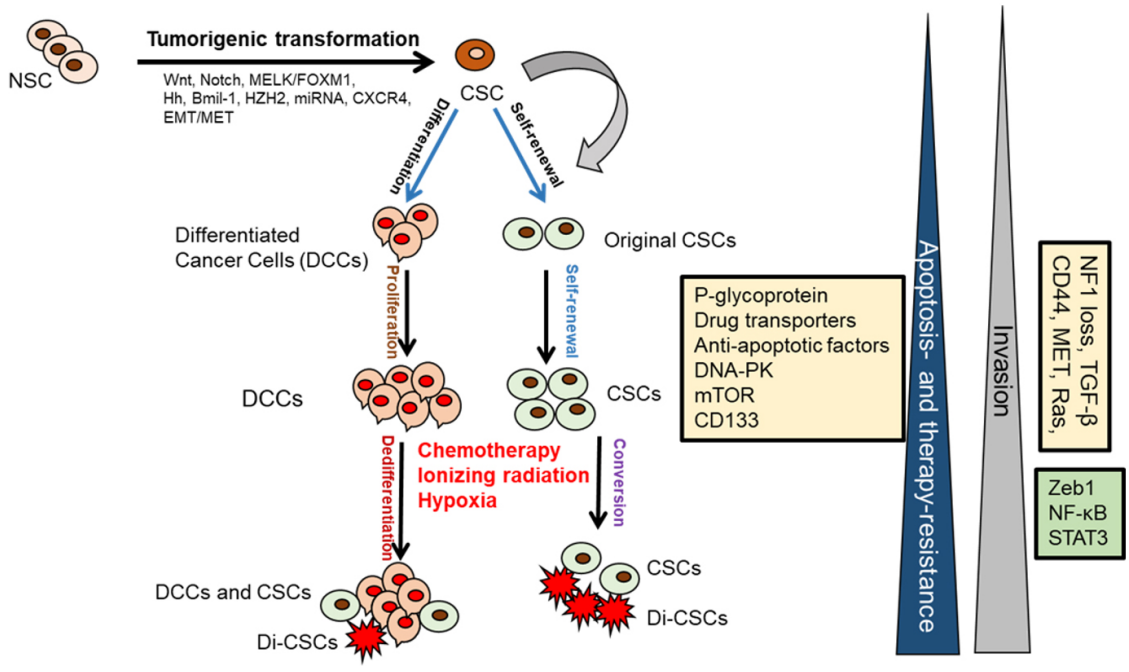

Figure 2. CSCs role in tumor development and progression. CSCs are originated from the NSCs through the tumorigenic transformation of several potential pathways including Hg, epithelial-to-mesenchymal transition (EMT), and the reverse process mesenchymal-to-epithelial transition (MET). CSCs and drug-induced CSCs (Di-CSCs) can be enriched following conventional chemotherapy treatment. CSC: Cancer stem cell; NSCs: normal stem cells.

subpopulations [Figure 1]. Another complexity of cancer treatment is that CSCs can be generated during cancer therapy by epigenetic plasticity due to drug-induced dedifferentiation and conversion of non-CSCs to CSCs[9,17,18] [Figure 2].

Tumor recurrence due to unresponsiveness to chemotherapy is the major cause of death in patients with incurable cancers and is due to treatment-resistant CSCs in the primary tumor [Figure 1]. CSCs are a low percentage of the cell population within a tumor and express specific molecular markers in a variety of cancers[14,15,17]. Understanding the molecular network of CSC populations may lead to the identification and development of targeted agents that can trigger CSCs cell death, thus enhancing the opportunity to design more effective treatment strategies to eradicate cancer. CSCs in various types of tumors are responsible for the initiation, progression, metastasis, drug resistance, and recurrence of cancer[9,14,15,17]. These quiescent and pluripotent cells form CSCs niches, resulting in particular microenvironments that protect CSCs from cell death, chemotherapy, and radiotherapy[9,18,19]. Additionally, tumors bear a hierarchy of cells initiated from the CSC population. Tumors exhibit stemness (self-renewal and multilineage differentiation) because of CSCs. These cells are capable of recapitulating xenografts similar to the original tumor[9,18,19]. The CSCs self-renewal and differentiation programming lead to the generation of several cancer cell types within tumors, creating tumor heterogeneity[9,18,19,23] with gradients of resistance to different therapeutics.

Drug resistance is a major impediment to the successful treatment of tumors with conventional chemotherapeutic agents[8,18,19]. One major contributor to drug resistance is the heterogeneity of cells with various degrees of sensitivity to drugs within a tumor[9,23]. A significant amount of data has proven that within solid tumors, there are distinct populations of cancer cells contributing to the complexity of cancer treatment[9,18,19,24-29]. Additionally, the lack of or refractoriness to apoptosis due to intrinsic resistance to cell death has been another primary limitation in cancer therapy (e.g., pancreatic cancer, colon cancer, glioblastoma, and prostate cancer are typically refractory to cancer chemotherapy mainly due to aberrant apoptotic machinery) along with acquired resistance (e.g., after breast cancer chemotherapy, tumor cells become resistant to multiple drugs)[18,28]. Based on substantial data, it is now believed that major contributors to intratumoral heterogeneity are CSCs, cellular genotype, genomic instability, cell plasticity epigenetic variation, and stochastic processes[9,18,19,29]. Additionally, the microenvironmental factors including distinct subpopulations of cancer-associated fibroblasts and cancer-associated macrophages[9,18,29], regulate various events in cancer cells and contribute to the heterogeneity of the tumor cell population. Therefore, while CSCs participate in drug and apoptosis resistance in tumors, the therapy resistance phenotypes in various cancers are very complex.

Various molecular and biochemical mechanisms participate in triggering resistance to chemotherapeutic drugs in cancer cells, and characterizing these mechanisms is critically important for the development and design of more effective and successful approaches to reverse or circumvent drug resistance in cancer cells and tumors. Upregulation of drug transporter proteins, deregulation of apoptotic signaling pathways, and upregulation of the cytoprotective and survival mechanisms in cancer cells, particularly in CSCs, confer resistance to various drugs in a wide variety of cancers[28,30-34]. Since several levels of drug resistance phenotype may be present in the bulk of tumor cell population, for effective and successful cancer therapy, it is essential to eliminate the entire CSC population, differentiated cancer cells, and progenitor cells in the entire tumor mass.

Drug resistance in CSCs

Several major signaling pathways have been shown to play essential roles in the regulatory capacity of CSC self-renewal, survival, proliferation, differentiation, and stemness maintenance. These pathways include

Janus-activated kinase/signal transducer and activator of transcription, Hh, Wnt, Notch, phosphatidylinositol 3-kinase/phosphatase and tensin homolog, and NF-κB signaling pathways[7-9]. It is also well documented that these critical signaling pathways are also dysregulated in various cancers[7-9,17,18]. Much evidence suggests that the dysregulation of these signaling pathways may also contribute to the survival and drug resistance of CSCs[18,19].

It is well documented that CSCs are highly resistant to conventional chemotherapies[11,26-32] and target specific anticancer agents. Figure 3 shows that various drug resistance mechanisms have been reported in CSCs including increased anti-apoptotic proteins such as Bcl-2 Bcl-X, and c-FLIP[11,26], high expression of ATP-binding cassette (ABC) transporter proteins and detoxifying enzymes[26-28], cell cycle quiescence[29,30], increased DNA repair ability[26,27], elevated aldehyde dehydrogenase (ALDH) activity[31], activation of key prosurvival signaling molecules such as Notch, Wnt/β-catenin, and NF-κB[32-34], increased activities of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR), and maternal embryonic leucine zipper kinase (MELK), aberrant stemness signaling pathways, increased quiescence, and increased autophagy[11,35].

Accumulating data show that CSCs are quiescent, which is the resting stage of the cell cycle, and quiescence is associated with resistance to chemotherapeutic agents since most of these drugs target actively proliferating cells[36,37]. DNA repair proteins are upregulated in CSCs, and their increased expression correlates with rapid DNA repair, which also triggers drug and radiation resistance[27,39,40]. Much evidence shows that the cancer microenvironment (niche) critically protects CSCs from cancer therapy[27,41], and CSCs mutually contribute to the niche in a feedback loop[32,41]. Furthermore, the extracellular matrix (ECM), a component of the niche, is known to facilitate and maintain CSCs and drug resistance[42]. Therefore, delineating molecular and biochemical mechanisms of drug resistance as well as understanding the cross-talk between CSCs and their niche is critical for devising strategies to overcome resistance to anticancer drugs and cell death.

This review article discusses the contribution of numerous drug resistance mechanisms and signaling pathways in controlling CSC maintenance and unresponsiveness to drugs and apoptosis. Understanding and delineating these mechanisms are critically important and essential for overcoming drug resistance in these cells[13-15,23,34]. To appreciate the complex signature network that controls unresponsiveness to drugs, the major mechanisms of chemotherapeutic and apoptotic resistance in CSCs are summarized in Figure 3. These mechanisms are interchangeable in controlling resistance to chemotherapy and apoptosis evasion in CSCs.

Signaling pathways in cancer stem cells

Significant evidence has documented that tumors are initiated from CSCs, and these cells maintain patient resistance to therapies[11,43-49]. Moreover, due to the heterogeneity, high diversity, and plasticity of CSCs, developing efficient and useful therapeutics to target these cells has been difficult. Accumulating data also suggests the possibility of non-CSC reprogramming and dedifferentiation of the progenitor cells or differentiated cancer cells to CSCs [Figure 1], resulting in increased complexity and diversity of drug-unresponsive cells with various drug resistance mechanisms in tumors. Therefore, because of this complexity, an ideally potent and effective anticancer drug must eradicate both CSCs and the bulk of the heterogeneous tumor cell population, and avoid triggering tumor cell dedifferentiation of non-CSCs to CSCs or cancer stem-like cells.

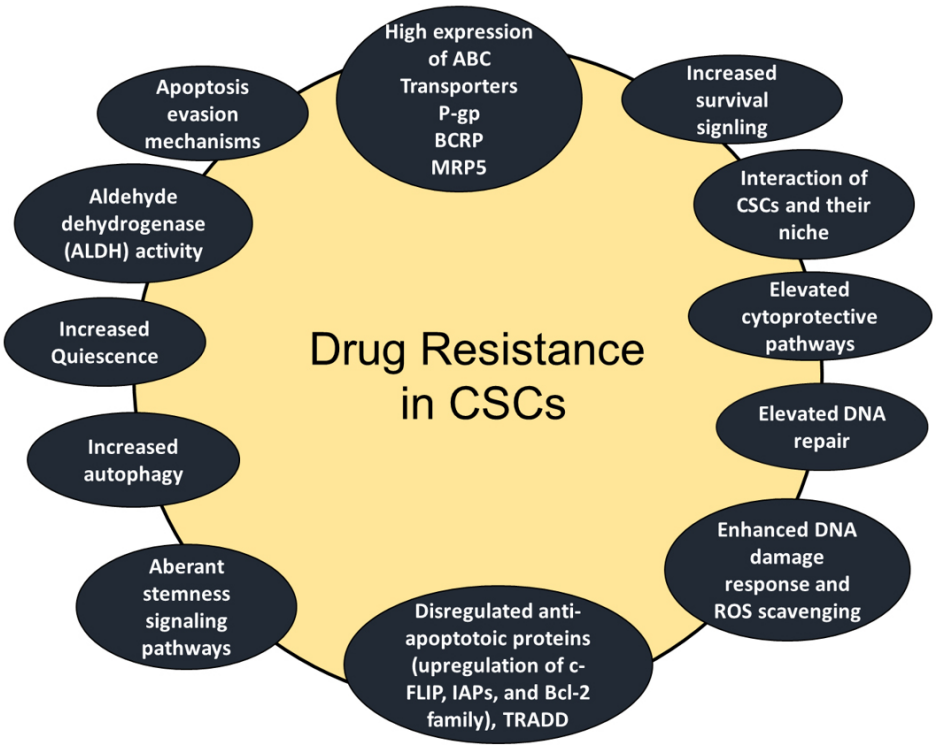

Figure 3. Schematic presentation of CSC-mediated therapy resistance to cancer. Activation of cell survival pathways, quiescence, increased drug efflux, impairment of the apoptotic pathway, increased DNA damage repair, increased detoxifying activity, and increased scavenging of free radicals are possible contributors to the therapy resistance of CSCs. TRADD**:** Tumor necrosis factor receptor 1 (TNFR1)-associated death domain protein.

A complex signature network including the Notch, Hg, Wnt/β-catenin, the NF-κB signaling pathways, PI3K/Akt/mTOR (mTORC1 and mTORC2), MELK, TGF-β, STAT, and Hippo-YAP/TAZ among others are activated and participate in the maintenance, self-renewal, proliferation, and drug resistance characteristics of CSCs[11,43-50]. These pathways and the cancer stem cell markers including CD133, CD44, Oct4, SOX-2, Nanog, and ALDH1A1 maintain distinct CSC properties[17,18,28,43-63] [Figure 4].

Accumulating evidence indicates that another important factor, epigenetic modification of CSCs, could result in phenotypic and functional heterogeneity among the cell populations within solid tumors which arise from different tissues of origin[9, 21, 23]. Emerging data suggest that epigenetic factors regulate CSC properties. For instance, the catalytic subunit of Polycomb repressive complex 2 (PRC2), known as the enhancer of zeste homolog 2 (EZH2), has histone methyltransferase activity, is upregulated in CSCs, and has a critical function in their proliferation and maintenance[61,64]. Furthermore, histone deacetylases (HDACs) 1, 6, 7, 8 and 6, known to deacetylate transcription factors and other cellular proteins, are overexpressed in CSCs and function in various maintenance activities of these cells[65,66].

It has been shown that hypoxia plays a crucial role in triggering resistance to chemotherapeutic agents[67-69]. Hypoxia-driven CSC enrichment results from a dedifferentiation process in breast cancer, and hypoxia-inducible factors (HIFs) are required for chemotherapy resistance in CSCs from various tumors including breast CSCs (BCSCs)[67], glioblastoma CSCs[68] and other solid tumors[57]. Interestingly, the dedifferentiated CSCs display multidrug resistance (MDR) via the PERK (protein kinase R-like endoplasmic reticulum kinase)-Nrf2 signaling pathway[70]. Moreover, Lee *et al.*[68] have found that temozolomide (TMZ)-triggered

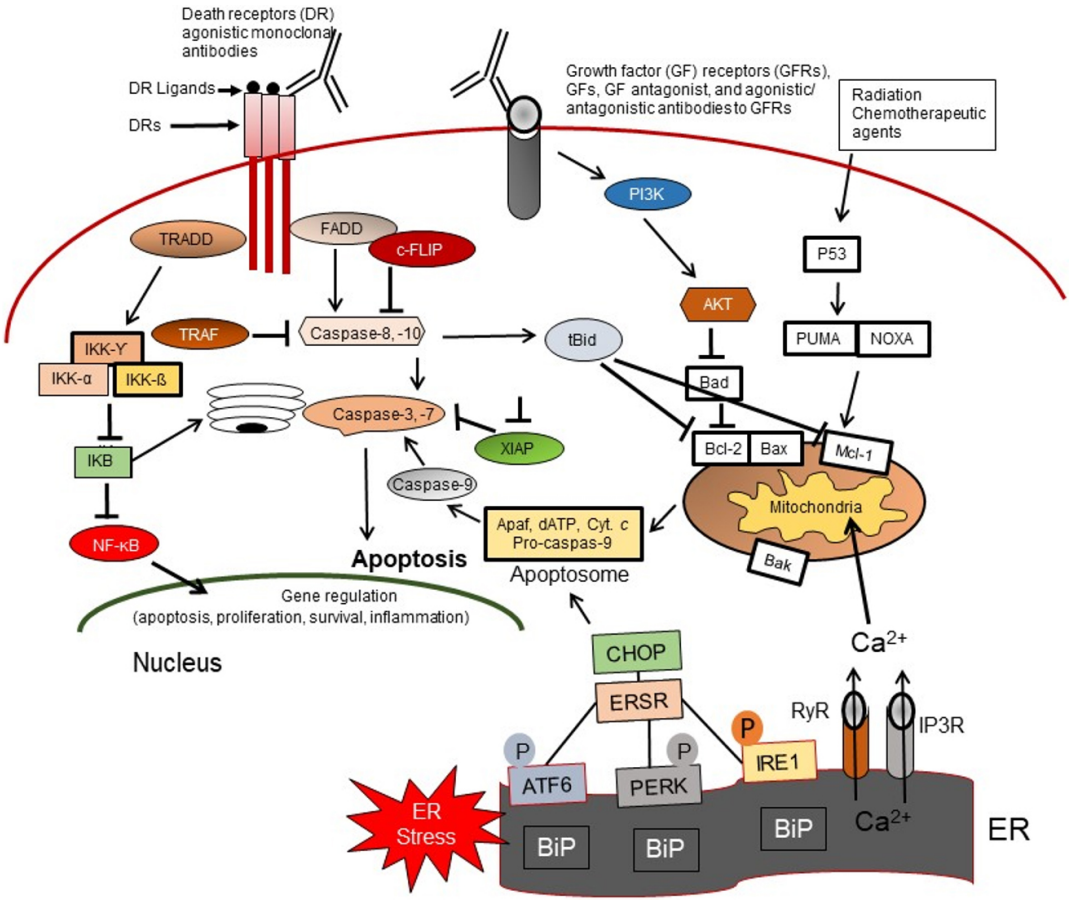

Figure 4. Apoptosis signaling pathways. Overview of the intrinsic (mitochondrial), extrinsic or death receptor (DR), and ER-stress (ERS)-mediated apoptosis pathways in response to the molecular action of anticancer agents, as well as the TRADD/NF-κB survival pathway, the growth factor (GF) receptors, and PI3K/Akt prosurvival signaling axis in CSCs. FADD: Fas-associated death domain; c-FLIP: cellular FLICE-like inhibitory protein; TRAF: tumor necrosis factor receptor associated factor; NF-κB: nuclear factor kappa B; IkB: inhibitor kappa B; IKK: inhibitor kappa B kinase; XIAP: X-linked inhibitor of apoptosis; Apaf-1: apoptotic Protease Activating Factor-1; Cyt. C: cytochrome c; PI3kinase: phosphoinositide 3-kinase; AKT: protein kinase B (PKB); PUMA: p53upregulated modulator of apoptosis; Bcl-2: B cell Lymphoma 2; Bax: Bcl-2-associated X protein; BID: BH3 interacting domain death agonist; Mcl-1: myeloid cell leukemia sequence 1; Bak: BCL-2-anatagonist/killer1; CHOP: C/EBP homologous protein; Noxa: encodes a Bcl-2 homology 3 (BH3) member of the Bcl-2 family of proteins; ATF: activating transcription factor; ER: endoplasmic reticulum; PERK: endoplasmic reticulum stress kinase; IRE1: inositol-requiring enzyme 1; RYR: ryanodine receptors Ca2+ release channels; IP3R: inositol 1,4,5-trisphosphate (IP3) regulated channels; BIP: binding immunoglobulin protein.

HIF1α/HIF2α upregulation plays a major role in converting non-stem glioma cells to stem-like cells, and that knockdown of HIF1α/HIF2α inhibited the conversion of non-stem glioma cells to glioma stem cells (GSCs) post-therapy[68].

Another critical signaling protein, MELK, a serine/threonine kinase, is upregulated in human cancers and CSCs[71-73], and evidence suggests that this protein plays a major role in the survival and other known properties of CSCs including drug and apoptosis resistance as well as tumor recurrence. Kim *et al.*[72] has shown that MELK phosphorylates the oncogenic transcription factor Forkhead Box M1 (FOXM1) and that the MELK/FOXM1 complex targets EZH2, which in turn promotes CSC resistance to drugs and radiation[18, 61], and that an inhibitor of MELK OTS167 robustly eliminates CSCs from small cell lung cancer (SCLC)[73,74].

Resistance to apoptosis in CSCs

While chemotherapeutic agents promote apoptosis in malignant cells and reduce tumor mass, the disease often relapses or progresses due to the repopulation of the cells unresponsive to anticancer therapy[75-77]. Moreover, cancer cells may acquire more stemness, metastatic properties, and drug resistance during treatment[78-80]. Therefore, this scenario suggests that therapy itself triggers tumor progression. Such unwanted effects of the therapies may be due to the selective survival of the particular subset of cancer cells having very aggressive mutations, allowing the cells to escape apoptosis[78], which may trigger tumor aggressiveness. However, this concept is challenged by data indicating a more complex scenario[81-86]. In fact, cancer tissues treated with cytotoxic agents work by aberrant responses through epigenetic mechanisms, activating signaling pathways directed towards tissue repair and cell repopulation. Such pathways also act by increasing tumor immune escape, metastasis, genetic instability, and acquired resistance to anticancer agents and apoptosis[87,88]. It is also possible that therapy-induced apoptotic cells produce paracrine signals, promoting proliferation capacity among surviving cells[89-93]. Therefore, the active role in a compensatory contradicting manner is played by the dying cells, which increase tumor tissue repopulation. In such a scenario, apoptotic cells activate the "Phoenix Rising" pathway to promote wound healing tissue regeneration (the term "Phoenix Rising" means to emerge from a catastrophe stronger and more powerful[89].

Unresponsiveness to chemotherapeutic agents, dysregulation of apoptosis pathways, apoptosis resistance, and overexpression of anti-apoptotic proteins are necessary for CSC survival. To discuss the mechanisms of resistance to apoptosis and cancer-related chemotherapeutic drug, apoptosis signaling pathways are first described. Cancer cells and CSCs avoid apoptosis, but apoptosis in these cells is carried out through several signaling pathways in response to chemotherapeutic agents and various apoptotic stimuli[28,95]. Mutations that occur in normal stem cells (NSCs) lead to the generation of CSCs [Figure 1], enabling them to evade apoptosis and leading to tumor formation[28,80].

A large amount of data has described three major apoptosis pathways: the extrinsic or cell surface death receptors pathway, the intrinsic or mitochondrion-initiated pathway, and endoplasmic reticulum (ER) stress-mediated pathway control of apoptosis [Figure 4][28,96-107]. Extrinsic or the death-receptor mediated apoptotic pathway is initiated by the binding of death receptors (DRs) with their ligands [interaction of Fas/Fas ligand, tumor necrosis factor-α (TNF-α)/TNF receptor 1 (TNFR1), TRAIL (TNF-related apoptosis-inducing ligand)/DR4, or TRAIL/DR5] [Figure 4]. Ligand and DR interaction induces recruitment of Fas-associated protein with death domain (FADD), also called MORT1, and procaspases-8 or -10 to form the death-inducing signaling complex (DISC), which by an autocatalytic process leads to activation of these procaspases to caspases-8 and -10. These initiator caspases subsequently activate the effector caspases-3, -6, and -7. Active forms of these caspases then trigger degradation of the downstream proteins leading to apoptosis. Caspase-8 or -10 cleaves the pro-apoptotic Bcl-2 family member Bid to truncated tBid, thereby linking the extrinsic apoptosis pathway to the intrinsic or mitochondrial pathway and inducing cytochrome *c* release from mitochondria[28,96,100]. The DR-initiated apoptosis pathway is suppressed by the anti-apoptotic protein cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), which inhibits DISC formation and activation of caspases-8 and -10 and blocks apoptosis[34,103,104].

In the intrinsic apoptosis pathway, various apoptotic stimuli (e.g., conventional chemotherapeutic drugs, DNA damaging agents, radiation, and small molecule anticancer compounds) induce mitochondrial outer membrane permeabilization (MOMP). MOMP induction is initiated by the activation of two groups of pro-apoptotic proteins: (1) the Bcl-2 homologous pro-apoptotic proteins (e.g., Bax, Bak, and Bad) and (2) the Bcl-2 homology domain-3 (BH3)-only family of proteins including Bid, Bim, and Puma[96-102]. Therefore, these proteins provide an interactive protein network with mitochondria, which leads to the release of

apoptosis triggering factors. The apoptosis-inducing factors (AIFs) include certain caspases, Smac/DIABLO, and other factors from the mitochondrial intramembrane space to the cytosol. Following release from mitochondria, cytochrome *c* and dATP bind to apoptotic proteinase-activating factor-1 (Apaf-1) to form the apoptosome, and this complex triggers procaspase-9 autoactivation. The active caspase-9 can activate caspases-2, -3, -6, -7, -8, and -10, leading to degradation of cellular proteins and resulting in apoptosis induction[28,102,103].

The third main apoptosis pathway is the endoplasmic reticulum (ER)-mediated apoptosis pathway [Figure 4]. One of the functions of the ER is to promote the correct folding of proteins. It also mediates ER-associated degradation of unfolded or misfolded proteins. Dysregulation of ER functions triggers an accumulation of unfolded or misfolded proteins in the ER lumen, resulting in ER stress (ERS), which triggers the unfolded protein response (UPR) or the ERS response (ERSR), leading to restored homeostasis or apoptosis[105-107].

Another mechanism by which CSCs display resistance to apoptosis is by upregulating the expression of anti-apoptotic proteins including the cellular FLICE-inhibitory protein (c-F1LIP), the Bcl-2 family of proteins, and inhibitor of apoptosis proteins (IAPs)[28,108,109]. CSCs upregulation of c-FLIP expression regulates resistance to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis[110]. Overexpression of IAPs also plays a crucial role in resistance to TRAIL and chemotherapeutic agents, as well as unresponsiveness to apoptosis[28,111].

Mechanisms of CSCs drug resistance

While CSCs are significantly resistant to drugs, there are several characteristics of these cells that may potentially help in the development of anti-CSC therapies. These characteristics include drug transporters, DNA repair machinery, specific cell surface markers, particular networks of transcription factor signaling, aberrant signaling pathways, epigenetic alterations, reprogramming and plasticity, interaction of CSCs with the microenvironment and CSC niche, and using specific metabolic pathways that regulate CSCs[55,71-73,93-95].

Several mechanisms trigger drug resistance and make CSCs refractory to apoptosis. Characterizing the mechanisms that evade apoptosis and identifying therapeutic targets to increase apoptosis in CSCs are particularly significant for successful cancer therapy. These mechanisms are discussed in detail in the following sections.

Multidrug resistance transporters in CSCs

Several ATP binding cassette protein transporters, including P-glycoprotein (P-gp, MDR1, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), breast cancer resistance protein (BCRP, ABCG2), and MRP5/ABCC5[14-120], have been extensively investigated as multidrug resistance transporters in various tumors. Overexpression of these proteins in several solid tumor types, AML, and myeloma leads to ATP-dependent efflux of a wide range of conventional chemotherapeutic agents. Overexpression of these proteins in the multidrug-resistant cells results in lower drug levels in the resistant cells, below the amount required to induce cell death[112-115]. Consistent with these observations, conclusive evidence shows that CSCs in various solid tumors and hematological malignancies upregulate these ABC transporters, resulting in drug resistance in these cells[116,117]. For example, Wang *et al.*[118] reported that Panc-1 pancreatic CSCs displayed resistance to gemcitabine, upregulated expression of CD133/CD44/Oct4/Nestin compared to the parental Panc-1 cells, and overexpressed P-gp and anti-apoptotic proteins. Moreover, in glioblastoma CSCs, epigallocatechin gallate (EGCG) treatment downregulated P-gp overexpression but not that of ABCG2 or O6-methylguanine-DNA methyltransferase (MGMT) and increased the cytotoxic effect of TM[118]. Additionally, Wilson *et al.*[116] demonstrated that ABCG5 in melanoma cancer stem cells (MCSCs) maintains

drug resistance and stemness in these cells. Therefore, the ABC multidrug transporter proteins are surface markers for CSC identification as well as their ability to transport drugs and enable CSCs to be resistant to drugs.

PI3K/Akt/mTOR signaling pathway plays a crucial role in CSCs

This is a critical pathway that functions in many important cellular activities and contributes to drug resistance in cancer. Several studies have clearly shown that upregulation of PI3K/Akt/mTOR plays a central role in the maintenance of CSCs[121-123]. Furthermore, emerging data suggest that this signaling pathway is a rational and promising target for developing anti-CSC drugs[119-125]. Indeed, some promising compounds targeting this pathway, including salinomycin, metformin, silibinin E1201, rottlerin, and torin, have been shown to be promising anti-CSCs therapeutics[120]. Additionally, the antidiabetic drug metformin, an inhibitor of PI3K/Akt/mTOR signaling, was shown to effectively reduce temozolomide (TMZ) resistance in CSCs[123]. Furthermore, the combination of metformin with the RAF inhibitor sorafenib also significantly decreased CSCs oxidative stress and drug efflux pump activity and synergistically killed these cells[124]. It is well known that CSCs heavily rely on mitochondrial oxidative phosphorylation[124]. Interestingly, metformin has been shown to use this metabolic weakness and increase CSCs sensitivity to many cancer chemotherapies, modulate drug resistance, and increase treatment efficacy[125].

Dysregulated anti-apoptotic Bcl-2 family proteins in CSCs

Distinct hallmarks of malignancies are apoptosis evasion due to dysregulation of signaling pathways and apoptotic proteins[28] and the ability of CSCs to self-replicate, proliferate, and metastasize[28,126]. While emerging data indicate that in various cancers, several steps within the extrinsic and intrinsic apoptotic pathways in CSCs may be dysregulated[28,126], the abnormal expression levels, as well as levels and ratios of pro-apoptotic and anti-apoptotic proteins and their contribution to drug resistance in CSCs have not been well described. Bcl-2 family proteins are well characterized and consist of the anti-apoptotic molecules Bcl-2, Bcl-XL, and Mcl-1 and the pro-apoptotic proteins Bax, Bak, Bid, Bim, Bik, Noxa, and Puma[128,129]. Increased levels of Bcl-2 family proteins were shown in CSCs, and high levels of these proteins have been shown to be associated with the apoptosis and drug resistance of CSCs[139,131]. This resistance is partly due to the ratio of anti- to pro-apoptotic protein levels, triggering the unresponsiveness of cancer cells to drugs and apoptosis, which enhances cell survival[28,130,131]. It has been shown that aberrantly overexpressed nuclear factor erythroid 2-related factor 2 (Nrf2), which is the redux-sensing transcription factor, promotes CSC survival by elevating transcription of the genes for drug transporters and the anti-apoptotic Bcl-2 proteins[132]. Due to the significance of expression of the Bcl-2 family of anti-apoptotic proteins for cell survival and resistance to apoptosis and drugs in CSCs[28,130-132], therapeutic interventions to eliminate CSCs using inhibitors are potentially an important strategy.

Role of NF-κB in CSCs resistance to apoptosis and drugs

Cancer cells and CSCs often display constitutively activated NF-κB expression that promotes levels of apoptosis inhibitory proteins and drug-resistant proteins, resulting in enhanced survival and resistance to therapies in cancer cells[134-137]. It is documented that the tumor necrosis factor receptor 1 (TNFR1)-associated death domain protein (TRADD) is an adaptor protein in TNFR1 signaling and participates in NF-κB activation as well as survival signaling in CSCs[136] downstream of DR4, DR5 [Figure 2]. Moreover, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promotes the formation of the intracellular Complex II composed of FADD, TRADD, caspases-8 and -10, RIP1, TRAF2 and IKK-γ[133]. Upregulated expression of TRADD activates NF-κB in glioblastoma (GBM) cancer stem cells (GSCs)[134]. Moreover, cytoplasmic TRADD is significantly associated with worse progression-free survival (PFS) in GBM patients[134]. Interestingly, knockdown of TRADD by shRNA in GSCs reduced NF-κB activity and triggered cell death in these cells, revealing that TRADD is required for the maintenance of CSCs

populations[134]. NF-κB signaling plays a pivotal role in the maintenance of CSCs[135] [Figure 4]. In ovarian cancer, INF-κB signaling supported by the RelB transcription factor directly regulates the CSC-associated enzyme aldehyde dehydrogenase (ALDH)[135]. Furthermore, the NF-κB signaling pathway plays a critical role in the drug resistance phenotype of gastric CSCs[135]. The NF-κB activity supports CSCs maintenance and reduces sensitivity to NF-κB inhibitors, indicating that high activity of NF-κB plays a critical role in the survival and drug resistance of CSCs[136,137].

Role of the anti-apoptotic IAP family in CSC drug and apoptosis resistance

The IAP family consists of survivin, IAP1, cIAP2, X-linked inhibitors of apoptosis (XIAP), ML-IAP, NAIP, and ILP-2[138-141]. IAPs suppress the activity of caspases-3, -7, and -9 and help cancer cells evade apoptosis[138,139]. Upregulation of IAP family proteins has been shown in various tumors and hematological malignancies and causes resistance to apoptosis, anticancer agents, and radiation therapy, as well as causing poor prognoses[148,139]. These proteins function through interactions of their BIR baculoviral IAP repeat (BiR) protein domains, and these interactions are antagonized by Smac/Diablo, a negative regulator for the inhibitors of IAPs and induction of apoptosis[138,139]. Intriguingly, survivin plays a role in CD133+ cell resistance of colon CSCs to 5-fluorouracil (5-FU), and a survivin inhibitor can be a potential new targeted agent against CD133+ colon CSCs[138].

The pivotal role of IAPs in maintaining medulloblastoma (MB) CSCs has been shown[139,140]. Therefore, the importance of IAP inhibitors with a preference for CD133+ positive MB CSCs has been demonstrated[139]. Evans *et al.*[141] has shown that XIAP drove constitutive NF-κB transcriptional activity in inflammatory breast cancer and maintained CSCs. Furthermore, Ji *et al.*[142] have found that XIAP has a critical role in maintaining CSCs in nasopharyngeal carcinoma (NPC) stem cells. These authors demonstrated that XIAP regulates SOX-2 stability of the CSC, which is important for the maintenance and self-renewal of NPC CSCs. Furthermore, Janzen *et al.*[143] showed the important role of IAPs in CSCs by demonstrating that the cIAP inhibitor B (Birinapant) overcomes platinum resistance in CSCs of ovarian cancer *in vivo*, revealing that IAPs may play a significant role in cancer drug resistance and recurrence.

c-FLIP regulates resistance to apoptosis and drugs in CSCs

The master regulator of the death receptor (DR) networks is c-FLIP. Besides its key role as an anti-apoptosis factor, c-FLIP may control necroptosis, pyroptosis, autophagy, nuclear factor κB (NF-κB) activation, and tumorigenesis[143-144]. c-FLIP is a catalytically inactive caspase-8/-10 homolog and a critical anti-apoptotic protein that suppresses cytokine- and chemotherapy-induced apoptosis and causes resistance to these agents[143]. c-FLIP is expressed as long (c-FLIP$_L$), short (c-FLIP$_S$), and c-FLIP$_R$ splice variants, which bind to FADD and/or caspases-8/-10 and TRAIL receptor 5 (DR5) and prevent DISC formation. Moreover, c-FLIP$_L$ and c-FLIP$_S$ are also known to have multifunctional roles in various signaling pathways, as well as activating and/or upregulating several cytoprotective and prosurvival signaling proteins including protein kinase B (PKB) or Akt, extracellular signal-regulated kinase (ERK), and NF-κB. Furthermore, the upregulation of c-FLIP is also induced by several kinases, including phosphatidylinositol-3 kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), and Ca2+/calmodulin-dependent protein kinase II (CaMKII)[34,143]. Several reports have shown that c-FLIP isoforms maintain the survival and resistance of CSCs to apoptosis and anticancer therapeutics[110,145,146]. CD133, a CSC marker that plays a role in CSC tumorigenesis, metastasis, and chemoresistance, can also upregulate the expression of c-FLIP in CD133+ cells, thus inhibiting apoptosis[147,148].

Aldehyde dehydrogenase activity

ALDH isoforms detoxify a variety of endogenous and exogenous aldehydes, and high ALDH activity has been frequently used as a selectable marker for CSCs[149,150]. Much evidence suggests that ALDH may be used

as a marker for CSC self-renewal, proliferation, differentiation, and resistance to drugs[149-151]. It is well documented that the ALDH protein family is a signature of CSCs and ALDH1A1 is the most studied ALDH isoform[121-122]. The expression of ALDH1 protein in CSCs is a negative prognostic indicator and predictor of poor clinical outcomes in cancer patients, and high ALDH activity has been attributed to chemoresistant CSCs in different tumor types[150-152]. In summary, substantial data indicate a critical role of ALDH, particularly ALDH1, in CSC biology and therapy resistance[149-152]. Therefore, inhibition of ALDH activity may be a rational and potentially useful therapeutic strategy for targeting CSCs with the aim of increasing the efficacy of cancer therapies.

Enhanced DNA damage response and ROS scavenging in CSCs

Much evidence has shown that CSCs are resistant to DNA damaging therapies by regulating the cell cycle, increasing DNA repair capacity, and effectively scavenging reactive oxygen species (ROS)[155-159]. DNA-damage response (DDR) is considered a significant source of resistance to DNA-damaging treatments and CSCs, and checkpoint inhibitors that sensitize CSCs to DNA-damaging treatments have been developed[158,159]. Interestingly, DDR appears as a relevant target to sensitize cancer cells and CSCs to conventional radio- and chemotherapies, as well as to overcome resistance[158,159]. Fang *et al.*[160] reported that in NSCLC, chemotherapy targeting DNA damage checkpoint (CHK1) signaling in CSCs was p53-independent and caused cell cycle arrest, more efficient DNA damage repair, and enhanced cell survival compared to the bulk of the tumor cell population. Moreover, targeting CHK1 and PARP1 may provide an effective anti-CSC strategy[157].

Autophagy as a cytoprotective and drug resistance mechanism in CSCs

Autophagy is a catabolic pathway that is characterized by autophagosome formation and triggers tumor cell survival and drug resistance[160-165]. Autophagy is critical as a survival mechanism in tumors with defects in apoptotic signaling pathways, and CSCs show a high level of autophagy which contributes to their survival and therapy resistance[131-133]. Autophagy also determines cell fate by targeting the degradation of key transcription factors, including p53 and FoxO3A, or by enforcing quiescent growth arrest[163]. Apart from promoting resistance to chemotherapy, high levels of autophagy in CSCs maintain their pluripotency, allow them to cope with low nutrients and hypoxia in the tumor microenvironment, regulate CSCs migration and invasion, and help them escape immunosurveillance[163]. Beclin 1, a Bcl-2 homology 3 (BH3) domain only protein, is an essential initiator of autophagy and a critical determinant of whether cells undergo autophagy or apoptosis[165]. The BH3 domain of Beclin 1 interacts with Bcl-2 family members. Therefore, the role of Bcl-2 in inhibiting apoptosis and autophagic cell death makes the Bcl-2 protein and autophagy manipulation excellent targets and strategies to inhibit drug, anti-apoptotic, and autophagy-related resistance mechanisms.

CSC dormancy, plasticity and drug resistance

Cellular dormancy refers to the phenomenon that cells are recruited into the G0-phase of the cell cycle but can enter cell division in response to mitotic stimulation[166-170]. Emerging data show that CSCs can mediate therapy resistance through dormancy[169]. Chemotherapy and radiation therapy are mainly effective against proliferating cells. Dormant tumor cells may be comprised of both CSCs and non-CSCs[170]. It has also been demonstrated that dormant cells express the transcription factor SOX-2, which is essential for their survival and resistance to therapy[171].

CSCs niche, TME and drug resistance

It is well-documented that the tumor microenvironment (TME) contains several components, including stromal cells, immune cells, cytokines, chemokines and growth factors, hypoxic regions, and ECM[18,172,173]. Tumor-associated macrophages (TAMs) play major roles in stimulating CSC self-renewal, angiogenesis,

and remodeling immunity, and creating a niche for CSC tumor invasion, metastasis, as well as plasticity and dynamic changes[174-177]. Additionally, the CSC niche modulates several signaling pathways leading to drug and apoptosis resistance including the Wnt/β-catenin, Notch, and Hh signaling pathways[176,177]. Moreover, TAM may control the main transcriptional regulators like Nanog, Oct4, and SOX-2 to maintain CSCs stemness[177,178].

Current evidence shows the complex interplay between the genes, epigenetic modifications, TME, and the EMT in CSCs plasticity. The CSCs plasticity results in the generation of different subpopulations of CSCs with varying molecular and biochemical traits leading to varied dissemination and drug-resistance phenotypes[18,179]. Adding to this complexity is the capacity of CSCs to dynamically switch to non-CSCs or to different subsets of CSCs, exhibiting significant metabolic plasticity[179]. Due to certain microenvironmental stimuli, some cancer cells may exhibit plasticity which results in resuming proliferation[175]. The CSC niche and reciprocal communications between the CSCs and the TME play a pivotal role in the initiation and development of the tumor[175]. The TME, in reality, brings together factors to trigger and amplify resistance mechanisms in CSCs. The TME is continuously exposed to nutritional, metabolic, and oxygen deprivation, which promotes CSC adaptation[44], leading to drug resistance. Drug resistance due to physical barriers to treatment and cell adhesion-associated drug resistance has been associated with the TME and CSC niche[177]. Novel treatment strategies targeting CSC niche-microenvironmental factors have been developed.

Targeting CSCs to overcome therapy resistance

Due to their drug and apoptosis resistance, as well as tumors and metastasis, CSCs significantly contribute to the unresponsiveness to cancer therapies, relapse, and adverse outcomes in cancer patients[18]. To reduce or eliminate CSCs and improve the patients' genes and prognosis, new therapies that target key signaling molecules targeting stem-associated proteins, inhibitors of the drug transporters, and transcription factors participating in CSC maintenance have been used or proposed[179].

Target deregulated CSCs signaling

Much evidence shows that the oncogenic functional role of CSCs is regulated by the dysregulation of several developmental signaling pathways in normal stem cells[18,46,180,181]. Since these dysregulated pathways participate in self-renewal, metastasis, and resistance to drugs and apoptosis in CSCs, targeting particular proteins in these pathways by small molecule inhibitors offers a novel approach for treating cancers displaying high rates of recurrence and therapy resistance[28,182]. Among strategies used to target CSCs, there are several compounds that target CSCs specific surface markers, the CSC microenvironment niche, and CSC signaling pathways, which are already undergoing clinical trials [Table 1]. In addition, some new anti-CSC immunotherapeutic approaches, such as chimeric antigen receptor T-cell (CAR-T) therapy, are expected to be an important method of eliminating CSCs[182]. Emerging data show that novel strategies targeting the CSCs-specific pathways are being pursued[183]. The small molecule inhibitors of such pathways alone and in combination with different therapeutic agents are in clinical trials[182-184]. For instance, combined treatment with cisplatin and the PI3K/Akt/mTOR pathway inhibitor BEZ235 compared with cisplatin alone significantly disrupted colony formation ability, triggered higher ROS levels, and induced higher levels of apoptosis in resistant ovarian cancer cells[185]. Additionally, this combination robustly inhibited the PI3K/Akt/mTOR signaling pathway, reversed EMT, and reduced CSC marker expression[185]. It has been demonstrated that the inhibition of ALDH activity by all-trans retinoic acid (ATRA) or the specific ALDH inhibitor diethylaminobenzaldehyde in breast CSCs (BCSCs) significantly increases the efficacy of doxorubicin, paclitaxel, and radiotherapy on triple-negative breast cancer (TNBC) cells[186]. Salinomycin (SLM), an ionophore antibiotic, has been shown to selectively kill BCSCs in various breast cancer subtypes by altering the expression of genes involved in metastasis-free survival, overall survival, decreasing tumorsphere formation, and EMT[187-190]. The combination of HA (hyaluronic acid)-coated SLM

Table 1. New drugs targeting CSCs in clinical trials[a]

CSC targets	Drug	Reference
BCL2	Venetoclax AT101	[202,203]
Notch	MK-0752 RF-03084014 Demcizumab	[204-206]
WNT	PRI-724	[207]
Hedgehog	Glasdegib	[208]
	Vismodegib	[209]
JAK	Roxolitib	[210]
PI3K	BYL719	[211]
EGFR	Bevacizcizumab	[212]
CXCR4	Plerixafor	[213]
FAK	Defactinib/VX-6063	[214]
MDR1	Dofequidar/MS-209	[215]
ABCG2		
EpCAM	Catumaxomab	[216]

[a]To locate the clinical trials using these drugs, refer to the reference numbers in this table.

nanoparticles and PTX nanoparticles showed the highest cytotoxicity against CD44+ cells[187]. Hence, combination therapy using a conventional chemotherapeutic drug and a cancer stem cell inhibitor could be a promising approach to overcoming cancer recurrence due to the resistant cell population[191]. CD44 has been shown to function as a hyaluronan receptor, and HA has been used to specifically direct drugs to the CSCs[192]. One study demonstrated that the use of hyaluronan-conjugated liposomes encapsulating gemcitabine significantly enhanced the efficacy of the drug against BCSCs and decreased the systemic toxicity of gemcitabine alone on normal tissue[194]. Another strategy used against CD44 is using antibodies that block the HA-binding site of CD44[194].

Dietary polyphenol compounds have been shown to act on self-renewal and survival pathways of CSCs. For instance, we have reported that sulforaphane (SFN) from cruciferous vegetables robustly inhibited the growth of GBM CSCs and was particularly effective in eliminating GSCs, which play a major role in drug resistance and disease recurrence[195]. SFN also has been shown to be strongly effective against CSCs from other types of cancer[196]. Other dietary compounds used to target and eliminate CSCs are epigallocatechin-3-gallate, catechin in green tea[102,103], resveratrol from red grapes and blueberries[196-198], curcumin[198], and piperine[200]. While these compounds and sulforaphane are very effective in eradicating CSCs, they are harmless to normal cells at the concentrations affecting CSCs, indicating that these compounds are appropriate candidates to be used in combination with conventional anticancer agents to robustly eliminate drug-resistant CSCs.

A list of the new drugs targeting CSCs in clinical Trials is shown in Table 1. The FDA has approved three new drugs that can target CSCs. These include (1) vismodegib, a Hg inhibitor that targets a subset of CSCs in basal cell carcinoma[201] and other solid tumors, such as esophageal cancer[202]; (2) the BCL-2 inhibitor venetoclax, which selectively eradicates AML stem cells and demonstrated that 60% of patients receiving it with other chemotherapy drugs had complete clinical responses[202]; and (3) AT101, another pan-Bcl-2 inhibitor [Table 1], targets CSCs and is effective in esophageal and gastric cancer patients[203]. Furthermore, in addition to the drugs listed in Table 1[202-216], a variety of FDA-approved repurposed drugs, which have

been used for various diseases, also target CSCs and improve treatment with current chemotherapeutic drugs[217]. These repurposed drugs include ones approved to treat diabetes (metformin and thiazolidinediones), parasitic diseases (chloroquine, niclosamide, mebendazole, and pyrvinium), psychotic disorders (thioridazine, clomipramine, and phenothiazines), alcoholism (disulfiram), lipid disorder (statins), inflammatory diseases (tranilast, auranofin, acetaminophen, and celecoxib), antibiotics (azithromycin), and other disorders. These drugs provide beneficial effects from combined use with conventional cancer therapies[217].

CONCLUSION

The foregoing discussion clearly demonstrates that CSCs are endowed with the signature properties of malignancy: self-renewal and replicative immortality; resistance to chemotherapeutic agents and apoptosis; EMT; invasiveness; metastasis; and tumor recurrence. The CSCs niche, multiple mechanisms of drug and apoptosis resistance in these cells, intra/inter tumor heterogeneity, and the complex interaction of CSCs with the TME render therapy very ineffective. Therefore, a greater understanding of these factors is needed for the emergence of novel and effective therapies which target CSCs as well as the bulk of tumor cell population. CSC-related drug and apoptosis resistance mechanisms may be important for predicting patient response to therapies and guiding treatment selection with contemporary anticancer drugs targeting CSCs and robustly eliminating the entire tumor mass in various tumors originating from different tissues. While a number of CSC-targeting drugs have been identified for cancer treatment, it is still too soon to determine the true usefulness of these agents in the clinical setting. A challenging task for the development of CSC-specific therapeutics is identifying and detecting specific biomarkers of CSCs, which can be used to analyze their population during tumor treatment. As discussed in detail, CSCs can contribute to tumor resistance to chemotherapeutic agents and apoptosis. However, studying them may provide a better understanding of the molecular mechanisms underlying CSC unresponsiveness to therapies and may lead to the identification of specific targeted therapeutics and novel strategies to increase the sensitivity of CSCs to cancer therapeutics. Such agents may be capable of eradicating CSCs and eliminating the bulk of tumor mass by themselves or in combination with other contemporary anticancer drugs.

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Conflicts of interest

The author declared that there are no conflicts of interest.

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Cancer Drug Resistance

Review

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Mechanisms of neratinib resistance in *HER2*-mutant metastatic breast cancer

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Abstract

Human epidermal growth factor receptor 2 (HER2) is a major drug target and clinical biomarker in breast cancer treatment. Targeting *HER2* gene amplification is one of the greatest successes in oncology, resulting in the use of a wide array of HER2-directed agents in the clinic. The discovery of *HER2*-activating mutations as novel therapeutic targets in breast and other cancers marked a significant advance in the field, which led to the metastatic breast and other solid tumor trials MutHER (NCT01670877), SUMMIT (NCT01953926), and one arm of plasmaMATCH (NCT03182634). These trials reported initial clinical benefit followed by eventual relapse ascribed to either primary or acquired resistance. These resistance mechanisms are mediated by additional secondary genomic alterations within *HER2* itself and via hyperactivation of oncogenic signaling within the downstream signaling axis.

Keywords: Neratinib, HER2, *ERBB2*, estrogen receptor, mutation

INTRODUCTION

Human epidermal growth factor receptor 2 (HER2)-positive breast cancers have long been treated with targeted therapy, comprising either monoclonal antibodies, such as trastuzumab or pertuzumab, which bind

to the extracellular domain of HER2, or tyrosine kinase inhibitors (TKIs), such as the reversible inhibitors lapatinib and tucatinib and the irreversible inhibitor neratinib[1]. Genome sequencing efforts have recently identified recurrent somatic mutations in the *HER2* (*ERBB2*) gene in HER2-negative (non-amplified) breast cancer. Recurrent *HER2* mutations have been proven to be oncogenic drivers in both preclinical experiments and clinical trials[2-9]. Activating *HER2* mutations typically fall into four categories, with distribution dependent on tumor type: single nucleotide variants (SNVs) in the extracellular domain, particularly S310F/Y; SNVs in the transmembrane domain; SNVs in the kinase domain; and small insertions in exon 20[4,10,11]. *HER2* mutations constitutively activate the tyrosine kinase receptor activity, leading to upregulation of downstream phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling[6,12]. *HER2* mutations are rare in primary cancers, occurring in 2%-12% of solid tumors depending on tumor type and disease stage. In breast cancer, *HER2* mutations vary in frequency from ~2% to 8% depending on disease stage and histology (higher in lobular)[7,10,11,13,14] and have been associated with poor prognosis[15,16]. The prevalence of *HER2* mutations is higher in patients with metastatic breast cancer (MBC) that has progressed after primary endocrine therapy (~6%), and these mutations have been causally associated with antiestrogen resistance[12,13,17]. Furthermore, *HER2* and estrogen receptor 1 gene (*ESR1*) mutations are mutually exclusive in primary breast cancer, suggesting that *HER2* mutations are independent predictive and prognostic markers in estrogen receptor (ER)-positive MBC[11,12,16].

Neratinib is an orally available, second-generation, pan-HER TKI that irreversibly binds to cysteine residues Cys773 and Cys805 in the ATP pocket of the tyrosine kinase domain of epidermal growth factor receptor (EGFR), HER2, and HER4[18]. Neratinib inhibits autophosphorylation, downstream signaling, and growth of EGFR- or HER2-dependent cell lines, with cellular half-maximal inhibitory concentration (IC_{50}) < 100 nM[18]. Neratinib has been approved by the United States Food and Drug Administration for use in patients with adjuvant and metastatic HER2-positive (overexpressed/amplified) breast cancer based on the results of the ExteNET and NALA trials, respectively[19,20]. Furthermore, neratinib has demonstrated significant anti-tumor activity in preclinical models of HER2-negative/non-amplified breast cancer and other solid tumors with *HER2* mutations[2,3,5]. In ER-positive, *HER2*-mutant cell lines, ER signaling was suppressed and cells were resistant to endocrine therapy via estrogen deprivation or fulvestrant treatment; sensitivity was restored upon exposure to neratinib[17]. Dual inhibition with neratinib and fulvestrant was required to inhibit the growth of ER-positive, *HER2*-mutant models[12], implying a need to inhibit both the ER and HER2 signaling pathways simultaneously.

Clinically, the utility of neratinib, alone or in combination with other agents, in patients with heavily pretreated *HER2*-mutant breast and other cancers was explored in the phase II SUMMIT and MutHER trials[4,7]. The SUMMIT trial demonstrated clinical benefit from single-agent neratinib in patients with several solid tumor types, including *HER2*-mutant breast cancers[4]. For patients with *HER2*-mutant, hormone receptor (HR)-positive MBC, both the SUMMIT and MutHER trials were amended to combine neratinib with fulvestrant to suppress both HER2 and HR signaling simultaneously. This dual combination was clinically active in heavily pretreated patients with *HER2*-mutant, HR-positive MBC, including those who had received prior fulvestrant and cyclin-dependent kinase (CDK)4/6 inhibitor therapy. In SUMMIT, the overall response rate (ORR) for neratinib monotherapy in patients with HER2-mutant, HR-positive MBC (n = 23) was 17.4%, while the ORR for neratinib plus fulvestrant (n = 47) was 29.8%, with clinical benefit rates of 30.4% and 46.8%, respectively[9]. Median progression-free survival (PFS) and duration of response (DOR) were also longer with the combination (3.6-month PFS and 6.5-month DOR for neratinib monotherapy; 5.4-month PFS and 9.2-month DOR for neratinib plus fulvestrant)[9]. In MutHER, results for neratinib plus fulvestrant (n = 31) were clinical benefit rate (CBR) of 30.0%-38.0% and PFS of 5.0-6.0 months[8,9], consistent with the enhanced inhibition observed preclinically[12,17]. The independent

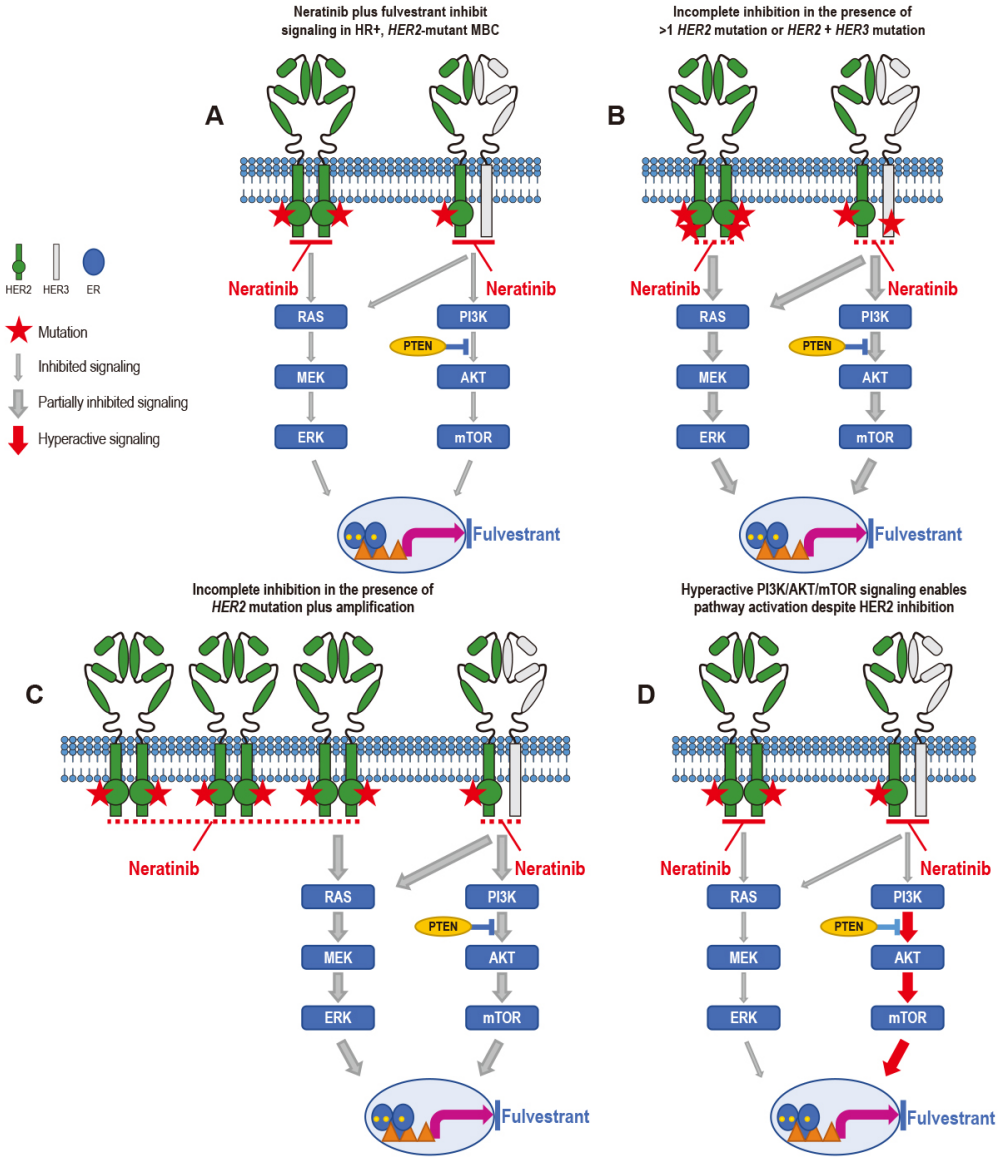

Figure 1. Mechanisms of resistance to neratinib. (A) Neratinib plus fulvestrant inhibit signaling in HR+, *HER2*-mutant MBC. In patients whose tumors harbor a single somatic activating mutation (red star) in *HER2*,neratinib strongly inhibits (thin gray arrow) HER2 pathway signaling, whereas fulvestrant inhibits ER signaling, leading to tumor growth inhibition. (B) Neratinib is less effective against/partially inhibits signaling in (thick gray arrow) tumors with more than one *HER2* mutation or *HER2* mutation plus *HER3* mutation or (C) *HER2* mutation plus amplification, whether these dual alterations are intrinsic or acquired. (D) Hyperactivation (thick red arrow) of downstream signaling can also preclude the effect of neratinib on mutant *HER2*.ER: Estrogen receptor; HR: hormone receptor; MBC: metastatic breast cancer.

PlasmaMATCH trial, in which patients with MBC were enrolled based on detection of an activating *HER2* mutation in circulating tumor DNA (ctDNA), reported that neratinib as monotherapy or combined with fulvestrant showed comparable clinical activity when patients were selected using this technique versus when the selection was guided by tissue testing, supporting the utility of ctDNA analysis in this patient population[21].

Unfortunately, patients who initially derived benefit from neratinib or neratinib plus fulvestrant in these studies eventually relapsed with metastatic disease, and a comparison of the genomic landscape of tumor tissue or ctDNA before treatment and upon progression revealed the acquisition of additional genomic aberrations[8,9,22]. Mechanisms of acquired resistance appeared to occur primarily via the development of secondary *HER2* genomic alterations (mutations or amplification), whereas intrinsic resistance was observed not only in patients whose baseline tumors had more than one *HER2* alteration, but also via alterations in the HER3/PI3K/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and MAPK signaling axis[8,9,22] [Figure 1].

MECHANISMS OF RESISTANCE

Accumulation of additional HER2 genomic events

Among patients with heavily pretreated MBC, the presence of more than one *de novo HER2*-activating event before treatment trended with a lack of benefit from neratinib alone or in combination with fulvestrant in trials to date. In the SUMMIT trial, six of the seven patients whose pre-treatment tumors harbored more than one *HER2*-activating event (second *HER2* mutation, $n = 2$; copy-number amplification, $n = 3$; or both, $n = 2$) did not derive clinical benefit[9]. In MutHER, three out of 48 patients had dual *HER2* mutations at enrollment; two of those three patients did not experience clinical benefit[7,8].

In patients whose treatment-naive tumors harbored one *HER2* mutation and who initially derived clinical benefit from neratinib-containing treatments, the one consistently observed mechanism of acquired resistance was the accumulation of a second (or further) additional *HER2* alteration[8,9]. In SUMMIT, three of nine patients with HR-positive, *HER2*-mutant MBC, who were treated with neratinib plus fulvestrant and who had both pre- and post-treatment tumors available for central sequencing, had additional *HER2*-activating events in the post-treatment tumor[9]. One patient had amplification of the mutant allele, one acquired a gatekeeper mutation, and one had amplification plus two acquired *HER2* hotspot mutations. Secondary *HER2* mutations were also detected in seven of 16 patients with paired pre-treatment and progression ctDNA who derived clinical benefit, including two of the three described above. Among patients in MutHER who had paired ctDNA samples, acquired *HER2* mutations were detected upon progression in three of six patients with clinical benefit following neratinib monotherapy, in four of seven patients with benefit following neratinib plus fulvestrant, and in one who experienced short-term stable disease[8]. Although several of the tumors acquired gatekeeper mutations (T798I and L785F)[23,24], the acquisition of additional sensitizing mutations or variants of unknown significance was also reported [Table 1]. Beyond *HER2*, no other acquired genetic event was consistently observed. These findings suggest that *HER2*-mutant MBCs are dependent on HER2 signaling even upon disease progression.

Aberrant HER3/PI3K/mTOR signaling

In preclinical models of HR-positive breast cancer, the recurrent *HER2* L755S and V777L mutations constitutively upregulated HER3 phosphorylation, particularly upon treatment with fulvestrant, resulting in hyperactivation of the HER3/PI3K/AKT/mTOR signaling axis and leading to antiestrogen resistance[12,22]. Structural modeling of the *HER2* L755S mutation revealed a loss of flexibility in the active state, allowing for increased HER2/HER3 heterodimerization and upregulation of PI3K/AKT/mTOR signaling[12]. *HER3* mutations have been modeled to stabilize HER2/HER3 dimerization and increase HER2 signaling[25,26], and preclinical models showed that dual *HER2/HER3* mutations further enhanced oncogenicity and promoted resistance to HER2-targeted therapies, including neratinib[26]. In SUMMIT, pre-existing concurrent activating *HER3* mutations were associated with poor treatment outcomes in patients with *HER2*-mutant MBC[9]. Further analysis of data from SUMMIT patients with *HER2*-mutant tumors across multiple tumor types revealed that mTOR pathway alterations were associated with a lack of clinical benefit with single-agent neratinib. Preclinically, hyperactivation of mTOR signaling was an actionable acquired mechanism of

Table 1. *HER2* alterations detected following neratinib-containing regimens in clinical trials of *HER2*-mutant MBC (compiled from the works of Ma *et al.*[7], Ma *et al.*[8], and Smyth *et al.*[9])

Trial	Regimen	*HER2* mutations detected at baseline	Best response	Acquired *HER2* alterations
MutHER				
	N	L755S, P761del	PR	R678Q, V697L
	N + F	G778_P780dup	PR	D808H[a], T798I[b], I767M
	N + F	S310F	PR	L755S, D769Y, G776V, T798I[b], L841V
	N + F	G778_P780dup	PR	S310Y, S310F, I767M, T798I[b]
	N	L869R; amplification	SD	D1011D[c]
	N	L869R, D769Y	SD	S310F, I767M, T862A, T798I[b]
	N + F	V777L	SD	S310F
	N + F	L755S	SD*	S310F
SUMMIT				
	N + F	S310F	CR	L785F[b,d]
	N + F	G778_P780dup	PR	I767M, S310Y, amplification[d]
	N + F	L869R	PR	S310Y, D769Y, L755S, T798I[b]
	N + F	V697L	PR	Amplified mutant allele[d]
	N + F	V777L	PR	T798I[b]
	N + F	L755S, L755P	SD	T862A, S310F
	N + F	G776V	SD	I767M
	N + F	L755S	PD*	D769H, D962H[a], K1171N[a], D1016Y[a], D1089Y[a]

All data are from circulating tumor DNA sequencing performed by Guardant360 for both MutHER and SUMMIT trials unless noted otherwise. All patients except those marked with an asterisk (*) achieved clinical benefit. [a]Variant of unknown significance. [b]Gatekeeper mutation. [c]Synonymous mutation. [d]Tissue samples, sequenced by Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). CR: Complete response; F: fulvestrant; MBC: metastatic breast cancer; N: neratinib; PD: progressive disease; PR: partial response; SD: stable disease.

resistance to neratinib in *HER2*-mutant cell lines and patient-derived xenograft (PDX) models[22]. Interestingly, however, *PIK3CA* mutations *per se* were not associated with a lack of clinical benefit with neratinib plus fulvestrant in *HER2*-mutant MBC[8,9], and no other single gene or mutation in the HER3/PI3K/AKT/mTOR signaling axis was clearly associated with acquired neratinib resistance.

Acquisition of somatic *HER2* mutations in HER2-positive breast cancer

Although this review focuses specifically on the acquisition of resistance to neratinib in HER2-negative, *HER2*-mutant MBC, the acquisition of *HER2* mutations in HER2-positive breast cancer also merits consideration. The co-occurrence of *HER2* mutations and amplification has been associated with poor response to trastuzumab and lapatinib, although neratinib has been shown to be effective against HER2-positive, *HER2*-mutant preclinical models and in patients whose breast tumors had coincident *HER2* amplification and mutation, suggesting neratinib as monotherapy may be effective in this setting[27]. In preclinical models of HER2-positive breast cancer, HER2 reactivation in lapatinib-resistant derivatives was driven by the acquisition of a *HER2* L755S mutation, which could be overcome by neratinib or afatinib[28]. Finally, recent findings from plasmaMATCH demonstrate that the incidence of *HER2* mutations in HER2-positive cancers increases with the number of lines of HER2-directed therapy[29]. Regardless of initial HER2-positive or *HER2*-mutant status, accumulation of genomic events within *HER2* itself is prevalent upon exposure to HER2-targeted agents. Whether or not there is a clinical difference in response depending on which type of alteration is the initial driver remains to be seen.

Taken together, these findings provide a rationale for the combination of multiple HER2 inhibitors or inhibitors of the downstream signaling axis in patients with *HER2*-mutant breast cancer, a therapeutic strategy that has already proven highly effective in HER2-positive breast cancer.

OVERCOMING NERATINIB RESISTANCE IN *HER2* –MUTANT MBC

The following are three possible approaches to overcoming neratinib resistance in *HER2*-mutant MBC: (1) dual HER2 targeting; (2) combination with PI3K, mTOR, MEK, or CDK4/6 inhibitors; and (3) sequential TKI treatment.

Dual HER2 targeting: neratinib plus monoclonal antibody or antibody–drug conjugate

The dual targeting of HER2 either upfront or at disease progression has proven to be effective in patients with *HER2*-mutant MBC. In MutHER, adding trastuzumab after disease progression on neratinib plus fulvestrant led to re-response in four of five patients, with a concomitant decrease in ctDNA[8]. In SUMMIT, the *HER2*-mutant breast cohorts were recently amended to treat patients upfront with the triple combination of neratinib, fulvestrant, and trastuzumab. This combination, in fact, demonstrated encouraging clinical activity in SUMMIT patients with heavily pretreated HR-positive, HER2-negative, *HER2*-mutant MBC who had previously received a CDK4/6 inhibitor (n = 33; ORR of 42.4%, CBR of 51.5%, median DOR of 14.4 months, median PFS of 7.0 months)[30,31]. Preclinically, neratinib combined with trastuzumab in *HER2*-mutant cancer models yielded more robust inhibition of HER2 signaling and growth than either agent alone[5,32].

Neratinib induces HER2 receptor ubiquitination and endocytosis[33]; combining neratinib with a HER2-directed antibody–drug conjugate may therefore enable increased payload internalization. In *HER2*-mutant PDX models, the combination of neratinib and trastuzumab emtansine (T-DM1) or trastuzumab deruxtecan (T-DXd) did, in fact, show synergistic tumor growth inhibition[34]. Safety and preliminary efficacy of neratinib plus T-DM1 have been demonstrated in patients with HER2-positive breast cancer[35]; clinical trials of neratinib plus antibody–drug conjugates are similarly warranted in the *HER2*-mutant MBC setting.

Combination with PI3K, mTOR, MEK, or CDK4/6 inhibitors

Combining neratinib with inhibitors of the downstream signaling axis or with CDK4/6 inhibitors may be a second approach to prolonging response to neratinib in patients with *HER2*-mutant MBC. First, preclinical data in *HER2/HER3* double mutant cell lines show that the combination of a PI3K inhibitor (alpelisib) with neratinib overcame neratinib resistance[26]. Second, the combination of the mTOR inhibitor everolimus with neratinib arrested the growth of neratinib-resistant, ER-positive, *HER2*-mutant organoids and xenografts[22]. Third, in two HER2-positive breast and colorectal PDX models harboring activating *HER2* mutations (V777L and R678Q) derived from patients who had been treated with HER2-targeted therapies, the combination of neratinib with the MEK inhibitor trametinib, the mTOR inhibitors everolimus or sapanisertib, or the CDK4/6 inhibitor palbociclib synergistically decreased tumor volume to a greater extent than any of the agents alone. These combinations were well tolerated in HER2-positive preclinical PDX models[36]. A clinical trial to study the safety and tolerability of neratinib combined with trametinib, everolimus, or palbociclib in metastatic solid tumors with HER family alteration or *KRAS* mutation is currently underway (NCT03065387)[37]. Given the promising efficacy of neratinib-containing regimens post CDK4/6 inhibitor in the SUMMIT trial[31], first-line treatment with neratinib plus a CDK4/6 inhibitor and endocrine therapy could warrant investigation if the combination is deemed tolerable.

Sequential treatment of neratinib with a second TKI

Sequential TKI treatment has long been standard in *EGFR*-mutant non-small cell lung cancer, and a similar approach could be investigated for *HER2*-mutant MBC. The *HER2* gatekeeper mutation T798I is recurrent in *HER2*-mutant MBC upon clinical progression following neratinib. Preclinically, another second-generation TKI, afatinib, and AZ5104, the metabolite of the third-generation TKI osimertinib, blocked *HER2* T798I mutation-induced cell growth and signaling[23]. These findings support the clinical investigation of sequencing TKI therapy in *HER2*-mutant cancers that develop gatekeeper mutations.

CONCLUSION

HER2-activating mutations are a targetable alteration in MBC and can be inhibited by neratinib. In heavily pretreated patients with MBC, more than one alteration in the HER2 signaling pathway, whether in the *HER2* gene itself or downstream in the signaling cascade, may preclude initial response. Furthermore, patients with a single *HER2* mutation who derive initial clinical benefit appear to become resistant via the acquisition of additional *HER2* mutations and/or amplification. Dual HER2-targeting via the addition of trastuzumab to neratinib, in combination with fulvestrant for patients with HR-positive MBC, has exhibited strong clinical activity against *HER2*-mutant MBC[8,30,31]; targeting both HER2 and CDK4/6 together may warrant exploration as part of a front-line approach in this setting. Future analysis of plasma samples from patients receiving dual HER2-targeting will elucidate whether acquired resistance occurs via the same mechanisms. Combination and/or sequencing of neratinib plus additional agents targeting either HER2 or downstream or alternative pathway members may be required for more durable clinical benefit. Any combination approach will require diligent clinical management given the gastrointestinal toxicity profile of neratinib, although neratinib dose escalation may help to mitigate adverse events[38].

Future studies may consider molecularly guided approaches beyond genomics, including but not limited to evaluation of changes in gene or protein expression or protein phosphorylation status, to inform the design of rational drug combinations and lead to improved outcomes for patients with *HER2*-mutant MBC.

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Authors' contributions

Conceived of this review article and compiled data from SUMMIT and MutHER publications: Eli LD
Made substantial contributions to data interpretation and manuscript writing: Eli LD, Kavuri SM

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Conflicts of interest

LDE is an employee and shareholder of Puma Biotechnology, Inc. SMK is a stakeholder in NeoZenome Therapeutics Inc.

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Cancer Drug Resistance

Opinion

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Improving HER2 testing reproducibility in HER2-low breast cancer

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Abstract

HER2 is a pillar biomarker in breast cancer, and it is assessed by immunohistochemistry (IHC) using a three-tier scoring system and reflex *in situ* hybridization (ISH) for IHC score 2+. Novel HER2-directed antibody-drug conjugates have demonstrated significant antitumor activity in breast cancers with low levels of HER2 expression, i.e. IHC score 1+ or ISH-negative IHC score 2+. Both primary and acquired resistance to anti-HER2 therapies remains a challenge in the treatment of breast cancers according to the HER2 positivity *continuum*. Thus, the ability to precisely discriminate among HER2-zero, HER2-low, and HER2-positive breast cancers is no longer a mere academic exercise. HER2 testing criteria, guidelines, and quality controls are re-gaining momentum for this new clinical need. Therefore, all preanalytical and analytical variables that might trouble the sensitivity and reproducibility of this test should be carefully considered to address all possible issues and open all possible therapeutic opportunities for breast cancer patients.

Keywords: Breast cancer, biomarkers, HER2, HER2 low, targeted therapy, antibody-drug conjugates, immunohistochemistry, ISH

INTRODUCTION: THE EXPANDED SPECTRUM OF HER2 POSITIVITY IN BREAST CANCER

HER2 status assessment is considered "the" predictive test in breast cancer pathology because of its

extremely relevant prognostic and predictive values[1]. Testing for HER2 consists of immunohistochemistry (IHC) using a three-tier scoring system and *in situ* hybridization (ISH) in case of IHC score 2+[2]. Using this method, patients with HER2+ breast cancer, i.e. IHC score 3+ or ISH-positive IHC score 2+ are eligible for anti-HER2 targeted therapy[3,4]. Recently, the DESTINY-Breast04 clinical trial demonstrated that targeting HER2 provides significant benefits also for patients with metastatic breast cancer showing low levels of HER2 expression, i.e. IHC score 1+ or ISH-negative IHC score 2+[5]. Of note, patients with hormone receptor (HR)+ disease had to be refractory/resistant to endocrine therapy (ET). In this randomized clinical study, the antibody-drug conjugate (ADC) trastuzumab deruxtecan (T-DXd) improved median progression-free survival by 4.8 months and median overall survival by 6.6 months, compared with standard single-agent chemotherapy. These data establish a new standard of care for patients with HER2-low breast cancer and a fast-track approval for T-DXd is expected. Indeed, these tumors were previously not eligible for HER2 targeting because they were considered resistant to HER2 inhibitor monoclonal antibodies[6]. Due to these groundbreaking advances, the capability to precisely discriminate among HER2-zero, HER2-low, and HER2+ breast cancers is no longer a mere academic exercise for pathologists. Instead, HER2 testing criteria, guidelines, and quality controls are re-gaining momentum to embrace this new upcoming clinical need.

THE EVOLUTION OF HER2 TESTING

Historical perspective

The American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) Expert Panel has made remarkable efforts to improve the analytical reliability of HER2 testing. Since 2007, they have provided guidelines on HER2 interpretation in breast cancer, including algorithms for defining positive, equivocal, and negative values for both HER2 protein expression and gene amplification. Specifically, a positive HER2 result was IHC staining of 3+ (i.e., uniform, intense membrane staining of > 30% of invasive tumor cells), a fluorescent ISH (FISH) result > 6 HER2 gene copies per nucleus, or a HER2 gene to chromosome 17 signals ratio > 2.2; a negative result was an IHC staining of 0 or 1+, a FISH result of < 4.0 HER2 gene copies per nucleus, or FISH ratio < 1.8[7]. Subsequently, in 2009, the intra-tumor heterogeneity of HER2 amplification was supplemented to the guideline and defined as HER2/CEP17 signal ratios > 2.2 in 5%-50% of the neoplastic cells[8,9]. Later, in 2013, to avoid false-negative results, the positivity threshold was decreased to 10% and the HER2/CEP17 ratio to 2[10]. With these updates, a considerably higher number of patients could be treated with trastuzumab with a reasonable, slight increase of adverse events. In 2018, ASCO/CAP released an integration to the 2013 edition, emphasized the coordination between IHC and ISH results, and mostly addressed specific clinical questions and technical issues related to HER2 equivocal and heterogeneity[3]. The current IHC algorithm with the expanded spectrum of HER2 reporting category and the reporting results for ISH assays are shown in Tables 1 and 2, respectively (Source: Breast Biomarker Reporting, CAP Cancer Protocol Templates, v1.4.1.1, November 2021 update, available at: https://documents.cap.org/protocols/Breast.Bmk_1.4.1.1.REL_CAPCP.pdf.

The evolution of different ISH types has established a proposal of reliable FISH alternatives with faster performance at a lower cost. Silver *in situ* hybridization and chromogenic *in situ* hybridization have demonstrated great reproducibility and have been approved by the U.S. Food and Drug Administration for HER2 testing in breast cancer. The combined technology of dual-color dual-hapten *in situ* hybridization (D-DISH), providing the convenience of light microscopy with an ISH specificity, using chromogenic probes instead of fluorescent ones, also demonstrates a high reproducibility[11]. Gene protein assays and next-generation sequencing are the newer modalities, which may potentially help to overcome the issue of the tumor heterogeneity, combining the benefits of both IHC and ISH by single-slide HER2 status assessment[12]. Other promising candidates for the alternative HER2 status assessment are molecular (multiplex ligation-dependent probe amplification) and liquid biopsy assays using circulating tumor cells,

Table 1. Reporting results of HER2 testing by immunohistochemistry and the corresponding expanded spectrum of positivity (ASCO/CAP 2021)

Score	Membrane staining pattern	Tumor cells	Classical category	Expanded spectrum
3+	Intense, complete (circumferential)	> 10%	HER2+	HER2+
2+	Weak-to-moderate, complete (circumferential)	> 10%	HER2+ if ISH+ and HER2- if ISH-	HER2+ if ISH+ and HER2-low if ISH-
	Intense, complete	≤ 10%		
1+	Faint/barely perceptible, incomplete	> 10%	HER2-	HER2-low
0	Faint/barely perceptible, incomplete	≤ 10%	HER2-	HER2 ultra low
	No staining		HER2-	HER2-zero

IHC: Immunohistochemistry; HER2: human epidermal growth factor receptor 2.

Table 2. Reporting results of HER2 testing by in situ hybridization (ASCO/CAP 2021)

Result	Assay	Criteria
Positive	Single probe	CN ≥ 6.0 CN ≥ 4.0 and CN < 6.0 and IHC = 3+ CN ≥ 4.0 and CN < 6.0 and dual ISH ratio ≥ 2.0
	Dual probe	Ratio ≥ 2.0 and CN ≥ 4.0 Ratio ≥ 2.0 and CN < 4.0 and IHC = 3+ Ratio < 2.0 and CN ≥ 6.0 and IHC > 1+ Ratio < 2.0 and 4.0 ≤ CN > 6.0 and IHC = 3+
Negative	Single probe	CN < 4.0 CN ≥ 4.0 and CN < 6.0 and IHC < 3+ CN ≥ 4.0 and CN < 6.0 and dual ISH ratio < 2.0 and CN < 4.0
	Dual probe	Ratio ≥ 2.0 and CN < 4.0 and IHC < 3+ Ratio < 2.0 and CN ≥ 6.0 and IHC < 2+ Ratio < 2.0 and 4.0 ≤ CN > 6.0 and IHC < 3+ Ratio < 2.0 and CN < 4.0

CN: Average HER2 copy number (signals/cell); IHC: immunohistochemical score; ratio: HER2/CEP17 ratio.

cell-free tumor DNA (ctDNA), and extracellular vesicles, which have been tested in IHC/ISH HER2-equivocal breast cancers. Transcriptomic analysis has revealed high *HER2* mRNA levels in HER2-enriched breast cancer subtypes using the PAM50 probe, suggesting another possible subtype signature. Dual HER2-blockade led to positive results in patients with higher *HER2* mRNA levels; thus, it is a newly proposed biomarker for chemotherapy de-escalation[13-15]. Despite evolving modalities of HER2 testing, they are currently awaiting approval and the IHC-ISH approach remains the "gold standard"[16].

HER2 targeting and biomarkers of drug resistance in breast cancer

The canonical HER2+ breast cancers account for 15%-20% of all breast malignancies and are characterized by the overexpression/amplification of HER2[9]. These patients can be treated with a variety of anti-HER2 compounds, including trastuzumab and pertuzumab, in both adjuvant and neoadjuvant settings[17,18]. Regrettably, the development of primary or acquired resistance is not uncommon in these patients[19]. Furthermore, despite the success of ET-combined therapy in the metastatic setting, a substantial proportion of initial responders eventually developing resistance and/or recurrence over the time. In this respect, novel predictive biomarkers are warranted. Several mechanisms have been proposed thus far, including phenotype shift through selective pressure[20-23]. Interestingly, it has been observed that low levels of HER2 expression, both in terms of the percentage of HER2-expressing cells and IHC staining intensity, may impair the binding of traditional anti-HER2 agents to the target[24]. An additional mechanism of resistance is represented by the activation of the phosphatidylinositol-3-kinase (PI3K)/Akt and the mammalian target of rapamycin (PI3K/AKT/mTOR) and cyclin D1-dependent kinase 4/6 (CDK4/6) pathways[25-27]. Finally, genetic alterations in HER2 regions responsible for kinase activation can compromise the ability of anti-

HER2 compounds to bind HER2. Specifically, studies have shown that mutations in positions L755S and T798I can cause the highest levels of resistance[28,29]. In this scenario, an appropriate and reproducible HER2 test able to precisely identify the various types of HER2 expression is essential to put in place the most appropriate HER2-targeted treatment combination.

Challenges and barriers for HER2-low identification

The current evidence on HER2-targeting therapies in HER2-low breast cancer arises from several translational research studies employing various classes of monoclonal antibodies, anti-HER2 vaccines, cellular immunotherapy, ADC, and bispecific antibodies[22]. In particular, ADC combines the selectivity of anti-HER2-targeted therapy with the cytotoxic potency of chemotherapy while reducing the systemic exposure to the cytotoxic payload[30-32]. Among this class of drugs, T-DXd is composed of a humanized anti-human HER2 antibody, an enzymatically cleavable peptide-linker, and a proprietary topoisomerase I inhibitor[30]. After the cleavage of the peptide-based linker by lysosomal cathepsins, the released cytotoxic molecules can affect HER2-low cells neighboring those with higher HER2 expression, reflecting a bystander effect; thus, T-DXd has shown the ability to target tumor cells regardless of their HER2-status[33,34]. These findings have transformed the traditional dichotomy of HER2 status and have raised the expectations in this field. However, whether HER2-low breast cancer represents a distinct biologic and prognostic subtype is still a matter of controversy. Recently, an analysis of 5,235 patients with HER2-negative invasive breast cancer revealed that HER2-low expression was positively associated with level of ER expression, and ER-low tumors were enriched among HER2-zero tumors[35]. The Authors of this study suggest that the worse prognosis of ER-low tumors might confound the prognostic analyses of HER2-low expression. Perhaps, re-testing and re-assess HER2 status in these retrospective studies, particularly for HER2-zero tumors would provide additional information. Assessment of data from CAP surveys and from a Yale University-based study of concordance of 18 pathologists reading 170 breast cancer biopsies revealed that the scoring accuracy for HER2 IHC in the low range (0 *vs.* 1+) was poor[36]. This inaccuracy might lead to misassignment of many patients for treatment with T-DXd. Further development of HER2-low breast cancer treatment includes combined treatment strategies evaluation, where high expectations rely on the ADC and immune checkpoint inhibitor combination[37].

Reducing false-negative and false-"low-positive" results

It has been reported that up to 55% of patients with breast cancer have a HER2-low disease[38-41]. However, up to now, discriminating between IHC score 0 with incomplete and faint staining in ≤ 10% of tumor cells and score 1+, as well as between score 1+ with faint staining and score 2+ with weak-to-moderate staining intensity, was a negligible pathology exercise[42,43]. In this context, updated guidelines for all the analytical phases are necessary to improve our diagnostic sensitivity for HER2-low breast cancer[43]. Although the ASCO/CAP continuously updates the guidelines to ensure optimal diagnostic performance, the interpretation of HER2 IHC in HER2-low breast cancer has not yet been formally endorsed[44]. Accurate and reproducible testing strategies and techniques are the key aspects to identifying patients who may benefit from ADC[43]. In this regard, to prevent false-negative/positive results and subsequent mistreatment, careful supervision of preanalytical and analytical issues is required[44]. A range of preanalytical factors such as fixation, antigen retrieval, antibody clones, enzymatic activity, reaction time, temperature, and substrate concentration may influence HER2 staining intensity[45,46]. Staining methodology, particularly antigen retrieval, the availability of diverse antibody clones with various specificity [i.e., PATHWAY® HER2 (clone 4B5; Ventana Medical Systems Inc., Tucson, AZ, USA), HercepTest™ (Dako Denmark A/S, Glostrup, Denmark), and Oracle® HER2 (clone CB11; Leica Microsystems GmbH, Wetzlar, Germany)] may significantly impact the accuracy and reproducibility of results and complicate the identification of HER2-low expression in terms of false-positive and false-negative results[46,47]. Test repetition for equivocal results may exclude possible technical problems; however, it does not often result in definitive positive or negative

results. Regarding the post-analytical phase, the lack of consistent epithelial internal positive control for HER2 within non-neoplastic breast tissue, HER2 intratumoral heterogeneity, particularly in HER2-low breast cancer, and the semi-quantitative and subjective mode of HER2 assessment may lead to inter-observer variability affecting the accuracy of the results[44,48,49]. A larger number of patients with HER2+ metastatic breast cancer might possibly have a long-lasting clinical response with the careful application of mor precise and reproducible diagnostic methods[50]. Thus, rigorous quality control procedures for specimen preparation coupled with well-defined guidelines for the precise assessment of HER2-low cases are required[51,52].

CONCLUSION

In light of the therapeutic armamentarium broadening based on HER2 patterns of expression, HER2 testing is becoming more complex. To improve sensitivity, specificity, and reproducibility of HER2 status evaluation, pathologists need to be aware of the molecular mechanisms underlying the variable expression of HER2 in different types of breast cancers and even in different cells within the same tumor. In this respect, continuous education, update, quality controls, and multidisciplinary discussions are key elements for optimal patient management.

DECLARATIONS

Authors' contributions

Wrote the initial version of the manuscript: Sajjadi E
Manuscript initially revised: Venetis K
Supervised and revised the paper and prepared the tables: Fusco N, Sajjadi E, Ivanova M

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Cancer Drug Resistance

Perspective

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Cancer-associated fibroblasts as accomplices to confer therapeutic resistance in cancer

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Abstract

The "seed and soil" concept has reformed paradigms for cancer treatment in the past decade. Accumulating evidence indicates that the intimate crosstalk between cancer cells and stromal cells plays a tremendous role in tumor progression. Cancer-associated fibroblasts (CAFs), the largest population of stroma cells, influence therapeutic effects through diverse mechanisms. Herein, we summarize the recent advances in the versatile functions of CAFs regarding their heterogeneity, and we mainly discuss the pro-tumorigenic functions of CAFs which promote tumorigenesis and confer therapeutic resistance to tumors. Targeting CAFs is emerging as one of the most appealing strategies in anticancer therapies. The endeavors to target or reprogram the specific subtypes of CAFs provide great cancer treatment opportunities, which may provide a better clinical benefit to cancer patients.

Keywords: Cancer-associated fibroblasts (CAFs), heterogeneity, therapeutic resistance, CAFs targeting

INTRODUCTION

Most conventional cancer treatment strategies are based on the special characteristics of tumor cells, such as rapid proliferation rate and oncogenic driver mutations in cancer cells. However, few patients experience complete responses, and the strength and duration of response to treatments vary widely. Intrinsic or acquired resistance developing during the treatment is the central issue in cancer therapies, which often leads to tumor progression. Thus, new therapeutic strategies are urgently needed to bypass or overcome drug resistance in cancer treatment. Before that, a profound understanding of the mechanism of resistance is needed.

The "seed and soil" theory, which Stephen Paget proposed first in 1889, has received widespread attention in recent years. Cancer-associated fibroblasts represent the majority of stromal cell populations in the tumor microenvironment (TME) and are closely linked with clinical outcomes across multiple cancers including colorectal cancer (CRC), pancreatic ductal adenocarcinoma (PDAC), and breast cancer[1-4]. While numerous studies have revealed that CAFs play pivotal roles in regulating tumor development and progression via various "intermediator messengers" involving extracellular matrix, soluble factors, and metabolites[5-7], bench-to-bedside translation remains the bottleneck for researchers to properly target CAFs as an efficient antitumor therapy. Thus far, no CAF-specific inhibitors have been approved by the United States Food and Drug Administration (FDA), and this might be, at least partially, ascribed to the high heterogeneity of CAFs.

Heterogeneity of CAFs origin

CAFs can be derived from various cell populations. Tissue-resident fibroblasts are considered one of the most prevalent precursors for CAFs. Soluble factors such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) derived from neighboring tumor cells have been implicated in de novo activation of CAFs[8,9]. Additionally, exosomes (including shuttling cargos such as miRNAs and lncRNAs) have also played essential roles in the transformation of normal fibroblasts (NFs) to CAFs. In some types of cancers, such as pancreatic and liver cancers, stellate cells are recognized as another critical source of CAFs, which have been termed pancreatic stellate cells (PSCs) and hepatic stellate cells (HSCs), respectively. Classic TGF-β, PDGF signaling, and vitamin A deficiency have been found to be involved in PSC activation[10,11]. Furthermore, a recent work unveiled that stimulation of IGF-1 signaling assisted HSCs in acquiring a fibroblast-like phenotype. Mesenchymal stem cells (MSCs) are also one of the most commonly studied sources of CAFs. Effectors stimulating transdifferentiation of MSCs to CAFs vary across different cancers[12,13]. For instance, the OPN-MZF1-TGF-β axis was able to mediate MSC-CAF transformation in breast cancer[14], while TGF-β, as well as CXCL16, participated in the activation of MSCs in prostate cancer. Lastly, other types of cells, including epithelial cells, endothelial cells, hematopoietic stem cells (HSCs), cancer stem cells, adipocytes, and pericytes, have also been reported to possess the potential to transdifferentiate into CAFs. Of note, there is less evidence relating to these origins, which needs further investigation[15]. Taken together, the origins of CAFs have not been fully elucidated yet. As distinct effectors/signaling pathways contribute to the generation of CAFs, which are related to cancer types or the cell types that the CAFs originated from, it would be meaningful to monitor the dynamic origins of CAFs more precisely during cancer progression by taking advantage of advanced technologies such as lineage tracing and single-cell special analysis.

Functional diversity of CAFs

Similar to the existence of heterogeneity in cellular origins, CAFs exhibit diversity regarding their biological characteristics and function, which was firstly corroborated by David A. Tuveson Group[16]. By exploiting in vitro 3D co-culture system and in vivo mouse/patient-derived PDAC tissues, they found two distinct subtypes of CAFs present in PDAC. One subpopulation of CAFs, located immediately adjacent to

neoplastic cells, showed elevated expression of α-SMA and low expression of IL-6 (myCAFs), whereas the other, distantly distributed throughout the tumor, had reduced α-SMA expression and elevated production of inflammatory factors including IL-6, thus was termed inflammatory CAFs (iCAFs). Intriguingly, these two subpopulations of CAFs showed distinct transcriptome profiles related to their characteristics and could dynamically change from one state to the other. This study highlighted that various subtypes of CAFs rather than one homologous pro-tumoral CAF population may exist in the TME, which can partially explain the failure encountered in clinical trials by targeting α-SMA+ CAFs[17,18]. Likewise, the two aforementioned CAF populations with distinct α-SMA expression and transcriptomes have also been reported in CRC, supporting the findings in PDAC[19]. Moreover, another study from the same group further identified a novel population of CAFs with MHC-II and CD74 expression termed antigen-presenting CAFs (apCAFs). This new subtype of CAFs can directly activate CD4+ T cells in an antigen-specific fashion, confirming the putative immune-modulatory capacity of CAFs[20]. In accordance, more and more in-depth studies have pointed to the functional diversity of these subpopulations of CAFs. For example, pharmaceutical inhibition or genetic ablation of Shh signaling, which is involved in driving myCAF activation in PDAC, resulted in increased metastasis and decreased animal survival[21,22]. A similar phenotype was demonstrated in parallel by another group, which also showed depletion of α-SMA+ fibroblasts and led to poorly differentiated tumors and shortened animal survival. More importantly, low myCAF content was found to be associated with worse overall survival in human PDAC tumor sections[21,23]. Collectively, this evidence strongly supports the tumor-constraining role of myCAFs, which should always be kept in mind when considering whether to target CAFs as anticancer therapy. On the contrary, accumulating evidence uncovers the pro-tumoral properties of iCAFs. This was not very surprising since the key hallmark of iCAFs is secreting inflammatory factors such as IL-6, which have been well-studied for their tumor-promoting capability[17]. Recent work from our lab showed that IL-6 secreted by CAFs can promote LRG1 expression through STAT3-mediated transactivation, which facilitates epithelial-to-mesenchymal transition (EMT) and ultimately leads to liver metastasis in a xenograft mouse model of CRC. Since many agents that target individual nodes of the IL-6/STAT3/LRG-1 cascade, including IL-6, IL-6R, or JAKs/STAT3, are currently under active investigations as treatments for hematopoietic malignancies and solid tumors, this work opens a new and implementable way to mitigate metastasis by blocking CAF-tumor cell crosstalk in CRC[24]. Additionally, another seminal study from David A. Tuveson Group revealed the underlying molecular mechanism that promotes the diversity of CAFs. They reported that IL-1 induced LIF expression and downstream JAK/STAT activation to generate iCAFs. Conversely, TGF-β was able to antagonize iCAF generation by downregulating IL-1R expression and promote shifting to myCAFs. Consistently, targeting JAK/STAT signaling reduced the number of iCAFs and increased α-SMA+ myCAFs, indicating a shift from iCAFs to myCAFs. Ultimately, this phenotypic shift within the two subpopulations of CAFs led to a dramatic decrease in tumor volume, confirming the opposite function of the two CAF subtypes. This study raised a promising strategy to tackle cancer by converting pro-tumoral CAFs to tumor-constraining CAFs or selectively depleting tumor-promoting CAFs[25]. The functional heterogeneity of cancer-associated fibroblasts in distinct tumors is summarized in detail in Table 1.

With advances in single-cell sequencing and multi-omics approaches, more and more novel CAF subsets have been unveiled across different cancers, which have been broadly described elsewhere[18,26]. Hereafter, to make the content of this review more clinically relevant, we mainly focus on discussing the CAF subpopulations with tumor-promoting properties, thereby possibly being considered as potential targets to overcome therapeutic resistance.

Conventional cytotoxic chemotherapies, targeted therapies, and the emerging innovative immune checkpoint inhibitors (ICIs) are the mainstays in treating cancer patients. Numerous studies have revealed

Table 1. Functional heterogeneity of cancer-associated fibroblasts in distinct tumor types

Cancer types	CAF subtypes	Characteristic markers	Functions	References
BC	CD10+/GPR77+ CAFs	CD10, GPR77	Chemoresistance, proliferation, migration	[34]
	dCAFs	*SCRG1, SOX9, SOX10, etc.*		[86]
	mCAFs	Fibulin-1, PDGFRα		[86]
	vCAFs/cCAFs	Nidogen-2	Angiogenesis	[86]
CRC	CAF-A	*MMP2, DCN, COL1A2, PDPN, FAP*		[88]
	CAF-B	*ACTA2, TAGLN, PDGFA, LUM*		[88]
OSCC	CAF-D	TGF-β1	Invasion, EMT	[90]
	CAF-N	Hyaluronan	Invasion	[90]
PDAC	apCAFs[a]	*H2-Aa, H2-Ab1, Cd74, Saa3, Slpi*	Antigen-present, Immunosuppression	[20,58,59]
	iCAFs[a,b]	*IL6, IL8, PDGFRA, CFD, PLA2G2A, HAS1, CXCL2, CCL2, CLU, EMP1, LMNA*	Immunosuppression, chemoresistance	[17,20,91]
	myCAFs[a,b]	*ACTA2, TAGLN, MMP11, MYL9, HOPX, POSTN, TPM1, TPM2*	Proliferation, migration, invasion, ECM remodeling	[17,20,91,92]
	meCAFs	Highly active glycolysis	Higher risk of metastasis and poor prognosis but better response to immunotherapy	[93]
	NetG1+CAFs	Netrin G1	Nutritional support (glutamate/glutamine metabolism), immunosuppression	[74]
PDAC/Oral/CRC/Bladder cancers	rCAFs	Meflin, BMP-4, Hedgehog, IKKβ	Antitumoral effect	[21-23,94-98]

BC: Breast cancer; CRC: colorectal cancer; OSCC: oral squamous cell carcinoma; PDAC: pancreatic ductal adenocarcinoma; EMT: epithelial-to-mesenchymal transition; ECM: extracellular matrix. [a]This is also found in BC; [b]this is also found in CRC.

the essential roles of CAFs in conferring therapeutic resistance through diverse mechanisms [Figure 1], such as remodeling of the extracellular matrix (ECM), maintaining the stemness of cancer stem cells (CSCs), and metabolic reprogramming[5-7,27]. More recently, growing evidence also demonstrates the ability of CAFs to modulate tumor immunity[15,28].

CAFs promote resistance to chemotherapy

Chemotherapies are still the main first-line treatment strategies for cancer patients. CAFs are one of the most prevalent stromal cell types within the TME across multiple cancers such as CRC and PDAC, and they play pivotal roles in regulating the response to chemotherapy. As the major source of extracellular matrix (ECM) components, CAFs are considered as a physical barrier to infuluence drug delivery. For instance, depletion of CAFs by inhibition of the Hedgehog cellular signaling pathway or administration of hyaluronan was able to enhance the delivery of chemotherapy (gemcitabine) in a PDAC mouse model[29,30]. Additionally, by upregulation of lysyl oxidase (LOX) or MMPs, CAFs can alter the abundance and composition of ECM components, especially collagen, ultimately leading to dysregulated ECM homeostasis and resistance to chemotherapy (epirubicin and paclitaxel)[31,32]. Cancer stem cells (CSCs) are a small cell population within the bulk tumors that possess self-renewing capability and are largely responsible for resistance to chemotherapy. Through the production of cytokines, chemokines, and exosomes (including shuttling cargos such as miRNAs, lncRNAs, or cirRNAs), CAFs play a pivotal role in regulating cancer stemness and are therefore also a route of therapeutic resistance[33]. A recent study revealed that IL-6 and IL-8 were secreted by a unique subset of CAFs expressing both CD10 and GPR77 against multiple chemotherapeutic interventions (doxorubicin, cyclophosphamide, and paclitaxel and docetaxel and cyclophosphamide) in breast and lung cancers. $CD10^{+}GPR77^{+}$ CAFs constantly secrete IL-6 and IL-8 through activating the NF-KB signaling pathway, providing a survival niche for cancer stem cells[34]. Our

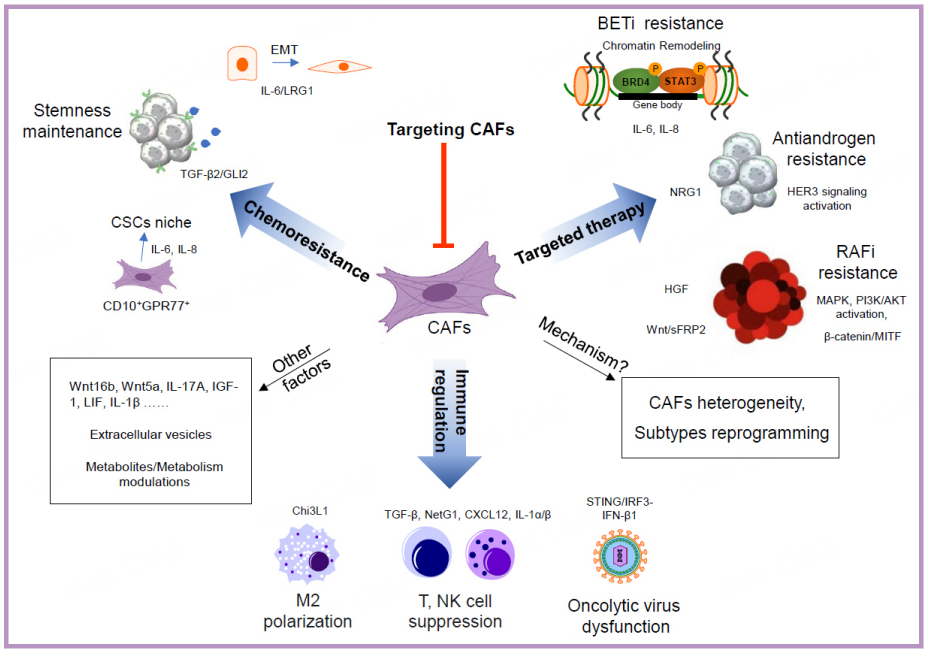

Figure 1. Roles of cancer-associated fibroblasts (CAFs) in cancer drug resistance. CAFs affect almost all cancer treatments, including traditional chemotherapy, targeted therapy, and immunotherapy. There are various mechanisms, such as secretion of growth factors, production of extracellular vesicles, and metabolites, through which CAFs promote drug resistance. Chemoresistance is enhanced by secreted factors from CAFs. Among them, TGF-β2 and IL-6/IL-8 from $CD10^+GPR77^+$ CAFs induce GLI2 upregulation and NF-κB activation, respectively, to maintain the stemness of cancers. IL-6 and/or IL-8 from CAFs were also found to induce EMT or chromatin remodeling in cancer cells. Upon stimulation with cytokines, such as IL-6, IL-8, NRG1, and HGF, the response to targeted therapies can be undermined by BRD4 modification, HER3 signaling activation, and MAPK and PI3K/AKT activation. Furthermore, CAFs mitigate tumor immunity by polarizing macrophages into the M2 phenotype, suppressing the function of NK and T cells and oncolytic viruses through Chi3L1, TGF-β, NetG1, CXCL12, IL-1α/β, and IFN-β1. Although appealing, targeting CAFs remains a big challenge today. The efforts to define CAF subtypes and further decipher their functions in the microenvironment may shed light on discovering new targeting strategies and provide more benefits to cancer patients. EMT: Epithelial-to-mesenchymal transition; CAF: cancer-associated fibroblast.

recent study also demonstrated that hypoxia-induced HIF1α and CAF-derived TGF-β2/Smad signal concurrently transactivate the expression of Hedgehog transcription factor GLI2 in cancer stem cells. Blockade of GLI2 signal induced by HIF1a and TGFβ2 effectively reverses the CAF-promoted resistance to 5-FU-based chemotherapy in colorectal cancer. Interestingly, a high level of HIF1α/TGFβ2/GLI2 can predict the high risk of recurrence in patients undergoing chemotherapy. This study proposed a potential biomarker as well as a potential new strategy to overcome chemoresistance by targeting CAFs signaling[35]. Similarly, by directly transferring exosomes as well as its shuttling cargo-miR-92a-3p to cancer cells, CAFs contributed to cancer stemness by activating the wnt/β-catenin pathway, which ended in resistance to therapy[36]. Of note, CAFs displayed a high basal level of autophagy compared to their counterparts in different cancers, such as PDAC, ovarian cancer, and head and neck squamous cell carcinoma (HNSCC), and autophagy could be further induced in response to stimuli from the TME[37-39]. It is believed that secretory autophagy promotes cancer survival by providing metabolites or other pro-tumoral effectors, including cytokines and growth factors in the harsh tumor milieu. It was also reported that the elevated level of autophagy in CAFs induced epithelial-to-mesenchymal transition and stemness in tumor cells, thus contributing to metastasis and drug resistance. Furthermore, another important role of stress-induced autophagy in CAFs was recently reported to regulate exosome release[40,41]. Thus, the involvement of autophagy in shaping the TME deserves further attention. Targeting autophagy-related core machinery by small molecules might be an effective alternative to deal with chemoresistance caused by CAFs.

CAFs enhance resistance to targeted therapy

In addition to chemotherapy, the CAF-derived secretome was also found to mitigate the response to targeted therapies. HGF was found to provide an alternative BRAF-independent mechanism for ERK-MAPK activation to mediate resistance to BRAF-targeted therapies in melanoma[42]. Wnt signaling modulated by CAFs has also been implicated in resistance to vemurafenib by attenuating the response of melanoma cells to DNA damage[43]. CAF-derived NRG1 confers antiandrogen resistance in prostate cancer by activating HER3 signaling[44]. In addition, we recently found that the CAF-derived IL-6/8-JAK2 signaling cascade can promote BRD4 phosphorylation[45], a critical epigenetic regulator in the regulation of cancer cell stemness. BRD4 phosphorylation induces chromatin remodeling, supporting a tumor-promoting transcriptional program and thus leading to BET inhibitor resistance. Given the prominent roles of epigenetic reprogramming in tumorigenesis and tumor progression, the finding paves a new way to more effectively treat CRC by co-targeting epigenetic modulators and CAF-mediated signaling pathways. In the presence of CAFs, tumor cells also displayed resistance to cetuximab, a monoclonal antibody therapy targeting epidermal growth factor receptor (EGFR). A further study ascribed this to increased secretion of EGF from CAFs[10,46]. Another recent study demonstrated that ECM remodeling and stiffness features were positively correlated with CAFs activation in CRC patients' tissue samples. Mechanistically, key components of the renin-angiotensin system (RAS), such as angiotensin II (ANGII) produced by CAFs, are involved in ECM deposition. More importantly, targeting CAF-derived RAS signaling was able to improve response to antiangiogenic therapy (bevacizumab), which was due to reduced ECM stiffness[47]. Intriguingly, matrix stiffness can induce autophagy in CAFs by stiffness sensing through the Integrin αV-focal adhesion kinase-AMPKα axis[48], forming a CAF–ECM positive feedback regulatory loop. Collectively, these studies show an intimate connection between CAFs and resistance to targeted therapy. Blocking CAF-related signaling pathways will be a powerful strategy to tackle this tough issue.

CAFs modulate response to ICI

Immunotherapy, specifically immune checkpoint inhibitors (ICIs), has led to a revolution in cancer treatment paradigms in the past decade. While ICIs have shown effectiveness in multiple cancers such as melanoma and lung cancer, the majority of patients cannot benefit from the treatment, especially those with "cold tumors", such as PDAC and CRC[49,50]. Based on the underlying mechanism of ICI action, several potential markers are proposed to be related to clinical response, including the PD-L1 expression level, specifically on tumor cells and APC cells, immune composition within the TME, neoantigens, tumor mutation burden, *etc*. Recent evidence shows that CAFs are linked to the resistance of ICIs[15]. CAFs can modulate the recruitment and activity of immune cells mainly through regulating ECM remodeling, the expression of immune checkpoints, and cytokines/chemokines, thereby skewing the TME to immunosuppressive status. For example, CAF-modified ECM is involved in the exclusion of cytotoxic T cells (CTLs) from the proximity of tumor cells. The secretion of matrix proteins and the production of matrix metalloproteinases (MMPs) by CAFs increased matrix stiffness, which not only promotes the migration and invasion of cancer cells but also serves as the physical barrier for immune cell infiltration[51,52]. Depletion of FAP+ CAFs, which exhibited upregulation of proinflammatory factors similar to iCAFs, can decrease tumor volumes in a CD4+ T cell- and CD8+ T cell-dependent manner in a KPC mice model[53,54]. Treating the FAP+ CAF-depleted mice with ICIs targeting PD-L1/CTLA-4 dramatically reduced tumor volumes. Furthermore, FAP+ CAFs are considered the principal source of CXCL12 and IL-6, which have been implicated in the prevention of T cell accumulation/activity in the tumor. Combined treatment with inhibitor targeting the CXCL12–CXCR4 axis or IL-6 antibody and anti-PD-L1 elicited synergistic efficiency in a PDAC mouse model[55-57]. apCAFs, which present antigens to CD4+ T cells through expressing MHCII molecules, were speculated to deactivate CD4+ T cells by inducing either anergy or differentiation into Tregs and dampen antitumor immunity[20,58]. Interestingly, an analog to apCAFs was reported to kill CD8+ T cells in an antigen-dependent manner via PD-L2 and FASL[59]. Thus, targeting

apCAFs might enhance antitumor immunity by restricting immune checkpoint activation. Another newly published work unraveled that CAF-derived wnt2 suppressed dendritic cell (DC) differentiation as well as DC-mediated antitumor T cell response. Targeting wnt2+ CAFs via monoantibody was able to significantly restore antitumor T cell response and enhance response to anti-PD-1 in both esophageal squamous cell carcinoma (OSCC) and a CRC mouse model[60]. Additionally, numerous studies have demonstrated that the expression of immune checkpoints such as PD-L1, PD-L2, and B7-H3 on CAFs can directly induce T cell exhaustion and deactivation[59,61,62]. Moreover, CAFs were reported to induce PD-L1, PD-1, cytotoxic lymphocyte-associated antigen-4 (CTLA-4), lymphocyte-activation gene-3 (LAG-3), and mucin-domain containing-3 (TIM-3) on the surface of immune cells or tumor cells, which dampen the proliferation and activity of immune cells, especially cytotoxic T cells[63-66]. By regulating the expression of those immune checkpoint molecules, CAFs also possibly potentiate the effect of ICIs. Enhanced recruitment of immunosuppressive cells, such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs), was also shown to reduce the sensitivity of immunotherapy[67-69]. For instance, overexpression of proline isomerase (PIN1) in CAFs was correlated with more infiltration of TAMs and fewer infiltrated CD8+ T cells in human PDAC tissue samples. Targeting pin1 rendered PDAC tumors more sensitive to anti-PD-1 treatment by disrupting the immunosuppressive TME[70]. CAF-derived soluble effectors such as CCL2 and CSF1 were critical for the recruitment of MDSCs. Depletion of MDSCs by targeting CSF1 was shown to significantly improve response to ICIs such as anti-CTLA4[53,67,71,72]. By taking advantage of single-cell analysis, a recently published seminal work has revealed a positive feedback loop between specific CAF-S1 clusters and Tregs in breast cancer, which subsequently contributed to resistance to immunotherapy[73]. In addition, a recently identified subtype of CAFs, termed NetG1+ CAFs, possessed intrinsic immunosuppressive properties and inhibited NK cell activity in PDAC[74]. Cardiotrophin-like cytokine factor 1 (CLCF1) derived from CAFs was able to promote infiltration and polarization of neutrophils in HCC[75]. Taken together, these studies suggest that co-targeting CAFs or CAF-derived signaling pathways might be one of the most attractive options to improve the efficiency of ICIs by reshaping the tumor immune microenvironment.

Challenges and Perspectives to target CAFs

Although tremendous efforts have been made to either directly or indirectly target CAFs [Figure 2], many strategies have failed to show promising clinical outcomes. The breadth of CAF functions and the interconvertibility of different subtypes pose a challenge for the field. In addition to their oncogenic functions, it has been revealed that CAFs can also play important roles in restraining tumors[21,23]. In several clinical or preclinical studies, targeting CAFs by some approaches did not lead to sufficient therapeutic efficacy or even promoted disease progression[76-78]. For example, depletion of α-SMA+ CAFs resulted in unexpected immunosuppression and aggressive tumor. Analogously, targeting the Sonic hedgehog (SHH) - smoothened (SMO) signaling that is involved in the activation of myCAFs also did not present therapeutic efficiency or, in some contexts, even shortened patient survival in clinical trials. Further studies indicated that this might be due to the aforementioned heterogeneity of CAFs present in the tumor milieu, and myCAFs tended to play a tumor-restraining role. On the contrary, depletion of FAP+ CAFs or interference with its derived CXCL12-CXCR4 axis restored antitumor immunity in PDAC. This prompted an ongoing phase II clinical trial involving patients with pancreatic cancer (NCT02826486). Thus, more efforts should be addressed to define CAF subtypes and further decipher their functions when interacting with other components in the microenvironment. In addition to direct depletion of CAFs, modulation of CAF activity would be another way to target CAFs. An important study described above found that the IL-1/JAK/STAT signaling cascade was mainly responsible for the generation of iCAF, which displayed tumor-promoting properties across multiple cancers. Targeting IL-1 or JAK was considered as an appealing approach to converting pro-tumoral CAFs into a tumor-restraining subpopulation. The preclinical data have encouraged an early phase I clinical trial to combine standard chemotherapy and IL-1 receptor antagonist

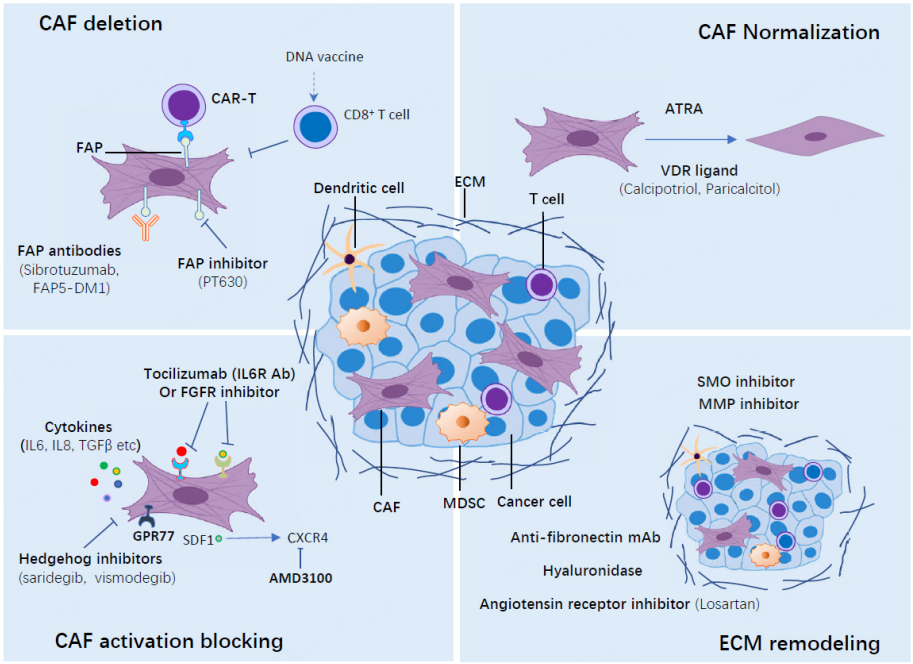

Figure 2. Targeting cancer-associated fibroblasts (CAFs) in cancer. Four main strategies targeting cancer-associated fibroblasts (CAFs) as cancer treatment are discussed. CAFs can be depleted by several treatments targeting CAF-specific markers, such as FAP and α-SMA. The normalization of CAFs from a pro-tumorigenic status to a quiescent or tumor-suppressive state can also be used for cancer treatment with small molecules such as ATRA or VDR ligands. The crucial signalings for CAF tumor-promoting function, such as cytokines and growth factors signalings, can be targeted to inactivate CAFs. Finally, CAF-derived extracellular matrix (ECM) proteins or related signalings can be targeted to induce ECM remodeling. MDSC: Myeloid-derived suppressor cell; FAP: fibroblast activation protein; CAR: chimeric antigen receptor; ATRA: all-trans retinoic acid; VDR: vitamin D receptor; FGFR: fibroblast growth factor receptor; SMO: smoothened; MMP: matrix metalloproteinase.

Anakinra in PDAC (NCT02021422). All-trans retinoic acid (ATRA) may normalize the CAFs to an inactive state in pancreatic ductal adenocarcinoma (PDAC)[79] (NCT03307148, NCT00001509). Pharmacological stimulation of the vitamin D receptor (VDR) with its ligand calcipotriol can induce stromal reprogramming, ultimately reversing chemotherapeutic resistance induced by CAFs in the PDAC models[80]. Targeting the crucial signalings for CAFs' tumor-promoting function, such as tocilizumab targeting IL-6 receptor[81] (NCT02767557) or an FGFR inhibitor[82] (NCT02699606, NCT01962532, NCT01703481, NCT02421185), can also be exploited to counteract the pro-tumoral effects of CAFs. Finally, CAF-derived extracellular matrix (ECM) proteins or related signalings can be targeted to induce ECM remodeling, which ultimately alleviates therapeutic resistance caused by CAFs. For example, losartan, an angiotensin inhibitor, can reduce hyaluronan production by CAFs, thereby improving vascular perfusion and drug delivery in breast and pancreatic cancers[83] (NCT04106856). The above-mentioned clinical trials targeting CAFs are summarized in Table 2.

The rapid development of single-cell RNA sequencing provides great opportunities to define subtypes and interpret the roles of CAFs. Although the high heterogeneity and diverse potential functions of CAFs have been revealed in many types of cancer utilizing scRNA-seq[20,84-88], there are still many details to be elucidated: (1) The precise roles of various CAF subtypes in therapeutic resistance remain largely undefined. The precise identification and characterization of CAFs' role in promoting or restraining tumors might pave new ways to target CAFs; (2) the mechanism of how the homeostasis between fibroblasts [including CAF subtypes and normal fibroblasts (NFs)] is maintained requires further investigation. For example, fibroblasts play different roles in tumors by secreting different cytokines[35]. The interconversion between

Table 2. CAF targeting strategies in cancers and related clinical trials

Target	Drugs/agents	Cancer models	Mechanism of action	Phase	Trail No.
CAF depletion					
FAP	FAP antibody (Sibrotuzumab)	Lung cancer	Depletion FAP^+ CAFs	Phase I	NCT02209727
	Talabostat (PT-100)	Multiple cancer types	Inhibits FAP enzymatic activity	Phase I-II	NCT00303940, NCT00086203, NCT00083252, NCT00083239, NCT00080080
CAF activation blocking					
Hedgehog	IPI-926 (saridegib) and GDC0049 (vismodegib)	Pancreatic Cancer	Reduced CAF activation	Phase I-II	NCT01130142, NCT01195415
	LDE225 (sonidegib)	Multiple cancer types	Inhibits Hedgehog signaling through SMO inhibition	Phase I-II	NCT02027376, NCT02195973, NCT02138929, NCT01487785, NCT01327053, NCT01708174, NCT01350115, NCT00961896
CXCR4	Plerixafor	PDAC, Ovarian and Colorectal Cancer	Inhibit CXCL12 production, restore antitumoral immunity	Phase I	NCT02179970
		Children Cancer, Solid Tumor		Phase II	NCT01225419
	BL-8040	PDAC		Phase II	NCT02826486
IL-6 receptor	Tocilizumab	Pancreatic Carcinoma		Phase II	NCT02767557
FGFR	JNJ-42756493 (erdafitinib)	Lymphoma, Adenocarcinoma, *etc.*	Prevents CAF activation	Phase I	NCT01962532, NCT01703481,
		Multiple cancer types		Phase II	NCT02699606
		Hepatocellular Carcinoma		Phase I-II	NCT02421185
CAF Normalization					
IL-1 receptor	Anakinra	PDAC	CAF normalization	Phase I	NCT02021422
Vitamin A metabolism	ATRA	PDAC, Nephroblastoma	Normalize stellate cells	Phase I-II	NCT03307148, NCT00001509
VDR	Calcipotriol	Breast Cancer,	CAF normalization	Phase I	NCT03596073
	Paricalcitol	Multiple cancer types	CAF normalization and improved chemotherapeutic efficacy	Phase I-II	NCT00637897, NCT03520790, NCT03883919, NCT03415854
ECM remodeling					
Angiotensin receptor	Losartan	Breast cancer, Pancreatic cancers	Reduces hyaluronan production by CAFs	Phase III	NCT04106856, NCT03900793, NCT01805453

PDAC: Pancreatic ductal adenocarcinoma; CAF: cancer-associated fibroblasts.

CAF subtypes might change the tumor's behavior. Furthermore, NFs can be educated to be tumor-promoting by tumor cells or CAFs[5,89]. Epigenetic and chromatin remodeling could be a potential mechanism to interpret these conversions. (3) The effect of therapeutic approaches on CAFs is another direction to be investigated. Many therapeutic paradigms such as chemotherapy could profoundly affect the CAFs' status and the tumor microenvironment in which they reside, ultimately changing the response of the tumors to treatments. With advances in technologies, the studies of multi-omics such as transcriptomics, proteomics, epigenomics, and metabonomics, improve our understanding of cancer biology in an unprecedented way. A comprehensive analysis and precise functional studies in CAFs are

required to integrate these multi-omics data using multiple model systems, especially at single-cell resolution. Next, super-resolved spatial omics studies may offer systematic approaches to understand the interplay between CAFs and other cells in tumors. In conclusion, these comprehensive investigations may warrant both preclinical and clinical studies targeting CAFs to achieve better clinical benefits for patients.

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Authors' contributions

Writing, review, and/or revision of the manuscript: Wang W, Cheng B, Yu Q
Conception and design of the perspective: Wang W, Cheng B, Yu Q

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Original Article

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Tyrosine kinases in KMT2A/MLL-rearranged acute leukemias as potential therapeutic targets to overcome cancer drug resistance

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Abstract

Aim: The main goal of this study was to elucidate at the transcript level the tyrosine kinase expression profiles of primary leukemia cells from mixed lineage leukemia 1 gene rearranged (KMT2A/MLL-R$^+$) acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) patients.

Methods: We evaluated protein tyrosine kinase (PTK) gene expression profiles of primary leukemic cells in KMT2A/MLL-R$^+$ AML and ALL patients using publicly available archived datasets.

Results: Our studies provided unprecedented evidence that the genetic signatures of KMT2A/MLL-R$^+$ AML and ALL cells are characterized by transcript-level overexpression of specific PTK. In infants, children and adults with KMT2A/MLL-R$^+$ ALL, as well as pediatric patients with KMT2A/MLL-R$^+$ AML, the gene expression levels for FLT3, BTK, SYK, JAK2/JAK3, as well as several SRC family PTK were differentially amplified. In adults with KMT2A/MLL-R$^+$ AML, the gene expression levels for SYK, JAK family kinase TYK2, and the SRC family kinases FGR and HCK were differentially amplified.

Conclusion: These results provide new insights regarding the clinical potential of small molecule inhibitors of these PTK, many of which are already FDA/EMA-approved for other indications, as components of innovative multi-modality treatment platforms against KMT2A/MLL-R$^+$ acute leukemias.

Keywords: AML, ALL, MLL gene, tyrosine kinase, Leukemia

INTRODUCTION

The lysine [K]-methyltransferase 2A (KMT2A)/mixed-lineage leukemia 1 (KMT2A/MLL) gene on chromosome 11 encodes a 431-kDa protein involved in the regulation of transcription[1]. Rearrangements (r) of the KMT2A/MLL gene have been reported in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)[2-14]. AML and ALL patients with KMT2A/MLL-R$^+$ leukemia have a poor prognosis with disappointing event-free survival (EFS) and overall survival (OS) outcomes on contemporary treatment regimens due to relapses caused by cancer drug-resistant clones[1-19]. Therefore, more effective frontline as well as salvage treatments for MLL-R$^+$ acute leukemias are urgently needed.

The main goal of the present study was to evaluate the protein tyrosine kinase (PTK) profiles of primary leukemic cells in patients with KMT2A/MLL-R$^+$ AML and ALL as potential therapeutic targets to overcome cancer drug resistance. We compared the transcriptomes of primary leukemic cells from KMT2A/MLL-R$^+$ *vs*. KMT2A/MLL-R$^-$ AML and ALL patients with an emphasis on the relative gene expression levels of for 21 PTK, including ERBB1, FGR, FLT3, FYN, HCK, JAK2, LCK, LYN, MERTK, SRC, BLK, BMX, BTK, ERBB2, ERBB3, JAK1, JAK3, PTK2, SYK, TEC, TYK2. Our studies provided unprecedented evidence that the genetic signatures of KMT2A/MLL-R$^+$ AML and ALL cells are characterized by transcript-level overexpression of specific PTK. In infants, children and adults with KMT2A/MLL-R$^+$ ALL, as well as pediatric patients with KMT2A/MLL-R$^+$ AML, the gene expression levels for FLT3, BTK, SYK, JAK2/JAK3, as well as several SRC family PTK were differentially amplified. In adults with KMT2A/MLL-R$^+$ AML, the gene expression levels for SYK, JAK family member TYK2, and the SRC family PTK HCK and FGR were differentially amplified. These results provide new insights regarding the clinical potential of small molecule inhibitors of these PTK, many of which are already FDA/EMA-approved for other indications, as components of innovative multi-modality treatment platforms against KMT2A/MLL-R$^+$ acute leukemias.

METHODS

Statistical methods for gene chip normalization for ALL and AML samples

A recently published working database, including data on primary leukemia cells from 201 adult patients with B-ALL (GSE13159), 119 pediatric patients with B-ALL (GSE11877 and GSE13351) and 97 infants with B-ALL (GSE68720), as well as 74 normal/non-leukemic control bone marrow samples (GSE13159), was built with previously archived datasets from the NCBI repository and used in our comparative gene expression analyses, as previously described in detail[20]. We also used a working database derived from archived datasets including data on primary leukemia cells from 542 adult patients with AML (GSE13159), and 279 pediatric patients with AML (GSE19577, GSE17855) along with the 74 normal/non-leukemic control bone marrow samples (GSE13159) in our comparative gene expression analyses, as described[20]. Probeset level normalization procedures were used as previously reported[20].

We also compared the gene expression profiles of primary leukemia cells from FLT3-ITD$^+$ pediatric AML patients (N = 48 from GSE17855) with those of primary leukemia cells from 189 FLT3-ITD$^-$ pediatric AML patients (GSE17855) as well as normal hematopoietic cells from 74 non-leukemic control bone marrow samples (GSE13159). Forty-seven patients from the KMT2A/MLL-R$^-$ "other" group of pediatric AML patients were FLT3-ITD$^+$, including 18 patients with cytogenetically normal AML, one patient with inv[16] AML, 12 patients with t(15;17) AML, 3 patients with t(8;21), 9 patients with AML and other cytogenetic features, and 4 patients who had AML with unknown cytogenetic features. One pediatric patient with KMT2A/MLL-R$^+$ AML was FLT3-ITD$^+$. This double mutant (FLT3-ITD/MLL-R) case was removed from

the analysis of FLT3-ITD$^+$ (N = 47; GSE17855) versus KMT2A/MLL-R$^+$ pediatric AML patients (N = 88; GSE17855, N = 46; GSE19577, N = 42).

No pediatric patients with KMT2A/MLL-R$^+$ AML harbored *NPM1* or *CEBPA* mutations. Within the KMT2A/MLL-R$^-$ ("other") group of KMT2A/MLL-R$^+$ AML pediatric AML patients, 17 harbored *NPM1* mutations (NPM1$^+$) and 16 patients had *CEBPA* mutations (CEBPA$^+$) (GSE17855). We investigated the gene expression profiles from primary leukemia cells from 71 KMT2A/MLL-R$^-$ pediatric AML patients who were FLT3-ITD$^+$, NPM1$^+$ or CEBPA$^+$ (GSE17855; only NPM1$^+$ N = 10, only CEBPA$^+$ N = 14, only FLT3-ITD$^+$ N = 38; 9 cases harbored one or more of the *NPM1*, *CEBPA* or *FLT3-ITD* mutations) versus 88 cases of KMT2A/MLL-R$^+$ [GSE17855 (N = 46); GSE19577 (N = 42)]. One patient with both FLT3-ITD and MLL-R was removed from this comparison.

Statistical methods for differential gene expression

Our analyses for ALL and AML focused on the expression levels (interrogated with 64 probesets) of the following 21 PTK genes: *ERBB1, FGR, FLT3, FYN, HCK, JAK2, LCK, LYN, MERTK, SRC, BLK, BMX, BTK, ERBB2, ERBB3, JAK1, JAK3, PTK2, SYK, TEC* and *TYK2*. Standard statistical methods, including mixed model ANOVAs, and hierarchical clustering method were employed, as reported[20-24].

RESULTS

Differentially amplified expression of PTK genes in primary leukemic cells from KMT2A/MLL-R$^+$ B-ALL patients

We first examined PTK gene expression profiles of primary leukemic cells from infants (N = 80), children (N = 25) and adults (N = 70) with KMT2A/MLL-R$^+$ B-ALL *vs.* normal hematopoietic cells from healthy volunteers (N = 74). Notably, FMS-like tyrosine kinase 3(FLT3) expression in infant KMT2A/MLL-R$^+$ ALL cases was 16.09-fold higher than in normal hematopoietic cells in non-leukemic control bone marrow samples (P-value < 1×10^{-8}) [Supplementary Figure 1; Supplementary Table 1]. The genes for several additional PTK showed augmented expression in KMT2A/MLL-R$^+$ infant ALL cells, including TEC, SRC, BLK, JAK2, BTK, PTK (3 probesets), SYK and JAK1 (2 probesets) [Supplementary Figure 1; Supplementary Table 1]. As the second most upregulated gene, the gene for BLK was expressed at a 6.77-fold higher level in infant KMT2A/MLL-R$^+$ ALL cells than in normal hematopoietic cells (P-value < 1×10^{-8}) [Supplementary Table 1]. Similarly, FLT3 wasexpressed at a 22.38-fold higher level in pediatric KMT2A/MLL-R$^+$ ALL cells than in normal hematopoietic cells (P-value < 1×10^{-8}) [Supplementary Figure 2; Supplementary Table 2]. As in infant leukemia cells, BLK was the second most upregulated gene in pediatric KMT2A/MLL-R$^+$ ALL cells showing a 4.38-fold higher expression level than in normal hematopoietic cells (P-value < 1×10^{-8}). The genes for several additional PTK showed augmented expression in pediatric KMT2A/MLL-R$^+$ ALL cells, including PTK2, TEC, BTK, and SYK [Supplementary Figure 2; Supplementary Table 2]. Similarly, FLT3 and BLK expression in leukemic cells from adult patients with KMT2A/MLL-R$^+$ ALL were 21.00-fold (P-value < 1×10^{-8}) and 21.79-fold (P-value < 1×10^{-8}), respectively, higher than in normal hematopoietic cells [Supplementary Figure 3; Supplementary Table 3].

We next compared the PTK gene expression profiles of leukemic cells from each KMT2A/MLL-R$^+$ subset to the *PTK* gene expression profiles of leukemic cells without KMT2A/MLL rearrangements from the corresponding control ALL patients in the "other" categories (Infants, N = 17; Children, N = 94; Adults, N = 131). In infants [Figure 1; Supplementary Table 4], children [Figure 2; Supplementary Table 5] and adults [Figure 3; Supplementary Table 6] with KMT2A/MLL-R$^+$ ALL, the gene expression levels for FLT3, BTK, SYK, JAK2/JAK1, as well as several SRC family PTK, including BLK, were differentially and significantly amplified. In KMT2A/MLL-R$^+$ infant ALL cells, FLT3_206674_at was the most significantly

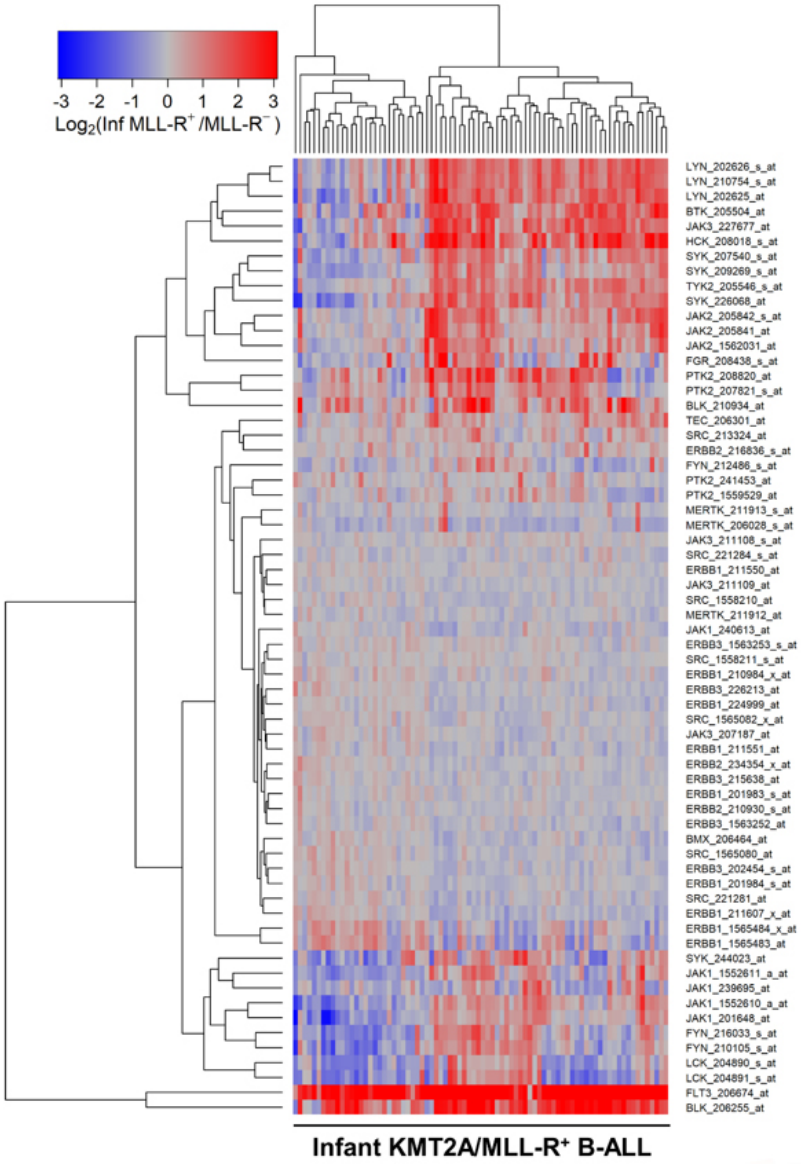

Figure 1. Gene Expression Levels for Tyrosine Kinases in Leukemic Cells from infants with KMT2A/MLL-R$^+$ B-ALL *vs.* Other Types of B-ALL without KMT2A/MLL Rearrangements. Probeset level normalized, robust multi-array analysis (RMA) signal values from the archived data for infant ALL (GSE68720) were examined in these comparisons. Infant KMT2A/MLL-R$^+$ B-ALL gene partners for KMT2A were AF4 (*N* = 48), ENL (*N* = 16), AF9 (*N* = 6), ASAH3 (*N* = 1), EPS15 (*N* = 3), Unknown (*N* = 6) (GSE68720; Total *N* = 80). The cluster figure displays the expression levels in KMT2A/MLL-R$^+$ ALL cells mean centered to the reference group [KMT2A/MLL germline/WT gene (KMT2A/MLL-R-)] represented by log_2-transformed fold change values (blue to red color indicates under-expression to over-expression respectively in KMT2A/MLL-R$^+$ samples). Co-regulated probesets are organized and depicted by dendrograms for both probesets (rows) and patients (columns). The log_2-transformed RMA values for leukemic cells from 80 infants with KMT2A/MLL-R$^+$ B-ALL compared to that from leukemia cells obtained from 17 infants with MLL-germline/WT ALL (MLL-R negative) showed 19 probesets that were upregulated in KMT2A/MLL-R$^+$ infant ALL cells. FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 11.23; *P*-value < 10^{-8}) followed by BLK_206255_at (Fold Change = 3.98; *P*-value < 10^{-8}) and HCK_208018_s_at (Fold Change = 2.46; *P*-value < 10^{-8}) [Supplementary Table 4]. Cluster visualization of the mean centered expression values suggested co-regulation of LYN (3 probesets), BTK, JAK3, HCK, SYK (3 probesets), TYK2, JAK2 (3 probesets), FGR, PTK2 (2 probesets) and BLK (2 probesets).

upregulated probeset (Fold Change = 11.23; < 10^{-8}) followed by BLK_206255_at (Fold Change = 3.98; < 10^{-8}) when compared to infant ALL cells with germline/wildtype KMT2A/MLL gene [Figure 1;

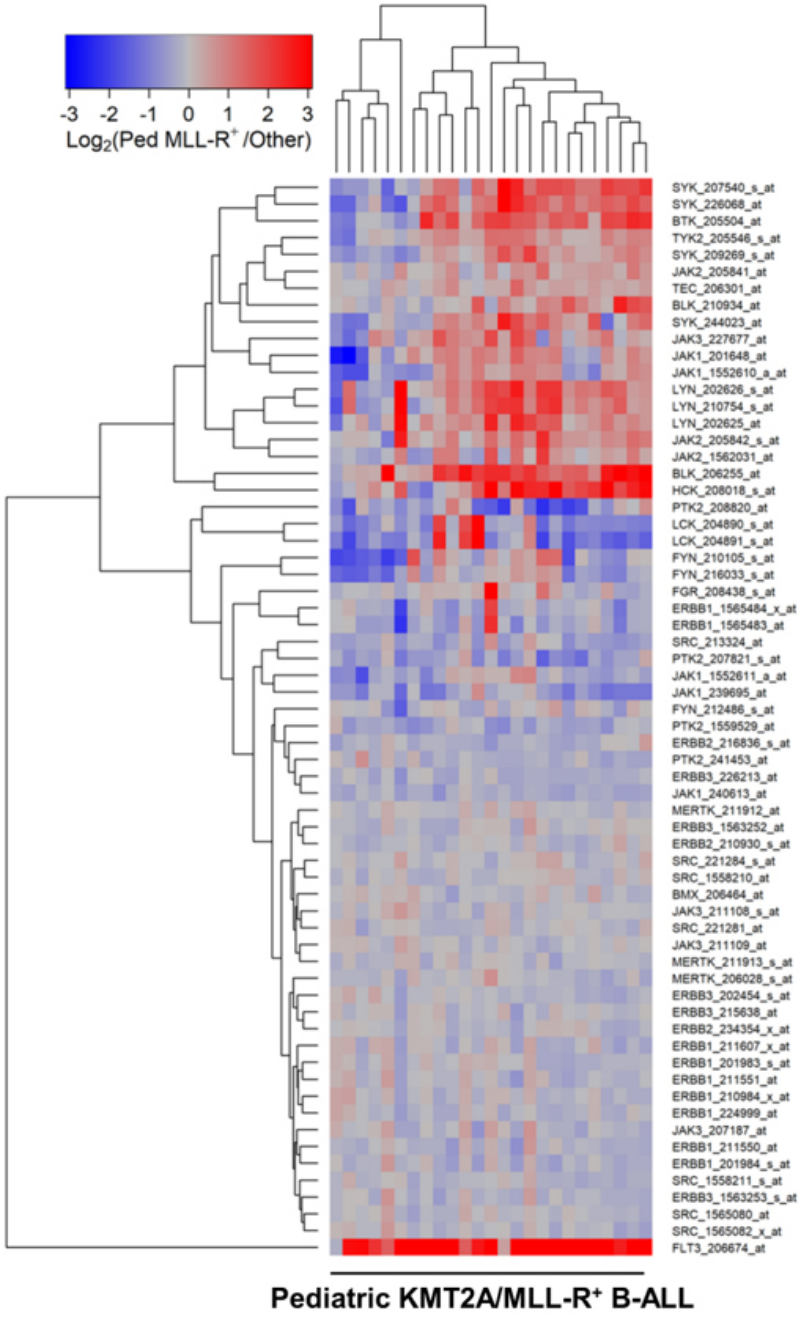

Figure 2. Gene Expression Levels for Tyrosine Kinases in Leukemic Cells from Pediatric Patients with KMT2A/MLL-R$^+$ B-ALL *vs.* Other Types of B-ALL without KMT2A/MLL Rearrangements. Probeset level normalized signal values from the archived data sets GSE11877 and GSE13351 were examined in these comparisons. The cluster figure displays the expression levels in KMT2A/MLL-R$^+$ ALL cells mean centered to the reference group (other subsets of ALL without KMT2A/MLL rearrangements) represented by $\log_2$-transformed fold change values (blue to red color indicates under-expression to over-expression respectively in KMT2A/MLL-R$^+$ samples). Co-regulated probesets are organized and depicted by dendrograms for both probesets (rows) and patients (columns). Depicted are the differential gene expression changes of $\log_2$-transformed, robust multi-array analysis (RMA) normalized values for 25 pediatric ALL KMT2A/MLL-R$^+$ cases (GSE11877 and GSE13351) compared to 94 non-MLL-R$^+$ other samples (GSE11877 and GSE13351) exhibiting 20 dysregulated probesets, of which 14 were upregulated in KMT2A/MLL-R$^+$ subset of cases. FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 8.65; *P*-value < 10^{-8}) followed by BLK_206255_at (Fold Change = 3.11; *P*-value < 10^{-8}) and HCK_208018_s_at (Fold Change = 2.64; *P*-value < 10^{-8}) [Supplementary Table 5].

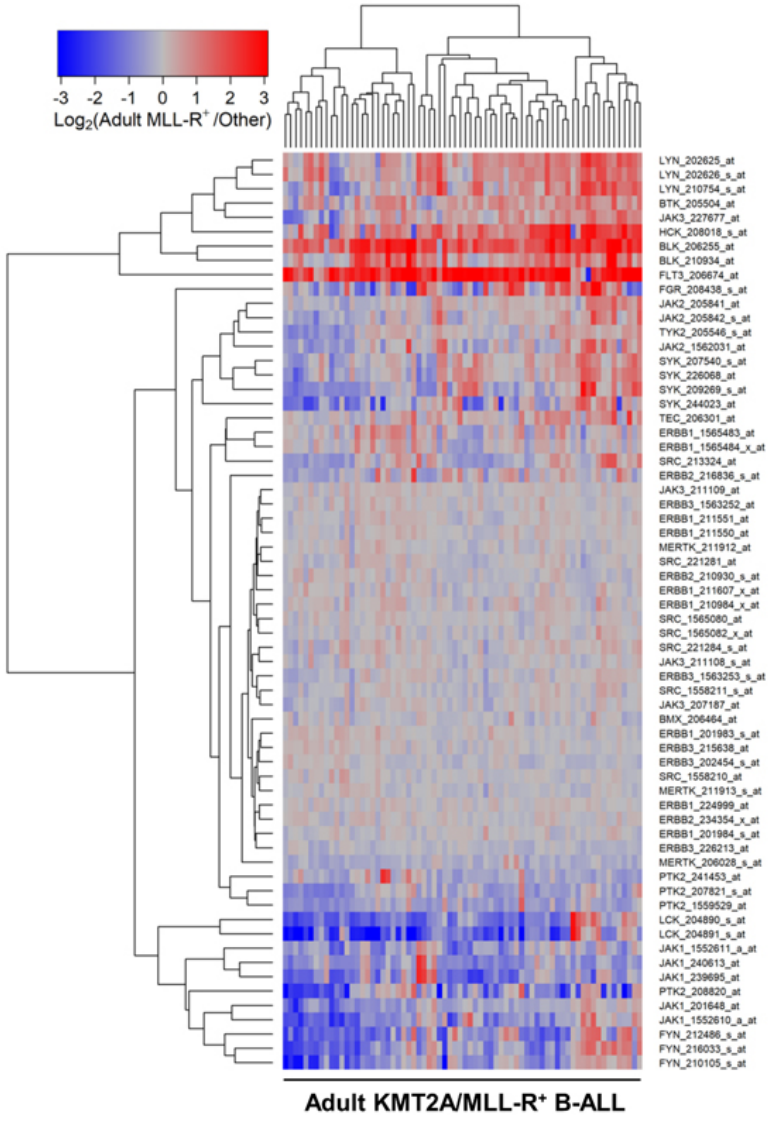

Figure 3. Gene Expression Levels for Tyrosine Kinases in Leukemic Cells from Adult Patients with KMT2A/MLL-R$^+$ B-ALL *vs.* Other Types of B-ALL without KMT2A/MLL Rearrangements. Probeset level normalized signal values from the archived data set GSE13159 were examined in these comparisons. The cluster figure displays the expression levels in KMT2A/MLL-R$^+$ ALL cells mean centered to the reference group (other types of ALL without KMT2A/MLL Rearrangements) represented by log_2-transformed fold change values (blue to red color indicates under-expression to over-expression respectively in KMT2A/MLL-R$^+$ samples). Co-regulated probesets are organized and depicted by dendrograms for both probesets (rows) and patients (columns). The comparison of the log_2-transformed RMA values for leukemic cells from 70 adult patients with KMT2A/MLL-R$^+$ ALL with the RMA values for leukemic cells from 131 adult patients with other forms of ALL resulted in 25 probesets that were significantly dysregulated, of which 10 were upregulated in KMT2A/MLL-R$^+$ subset of cases. FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 5.69; *P*-value < 10^{-8}) followed by BLK_206255_at (Fold Change = 3.95; *P*-value < 10^{-8}) and HCK_208018_s_at (Fold Change = 2.21; *P*-value < 10^{-8}) [Supplementary Table 6]. FLT3 was co-regulated with BLK (2 probesets), HCK and LYN (3 probesets).

Supplementary Table 4]. Likewise, in KMT2A/MLL-R$^+$ pediatric ALL cells, FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 8.65; *P*-value < 1×10^{-8}) followed by BLK_206255_at (Fold Change = 3.11; *P*-value < 10^{-8}) when compared to pediatric ALL cells without KMT2A/MLL rearrangements [Figure 2; Supplementary Table 5]. Similarly, in KMT2A/MLL-R$^+$ adult ALL cells, FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 5.69; *P*-value < 10^{-8}) followed by BLK_206255_at (Fold Change = 3.95; *P*-value < 10^{-8}) when compared to leukemic cells from

adult ALL patients without KMT2A/MLL rearrangements [Figure 3; Supplementary Table 6].

Differentially amplified expression of PTK genes in primary leukemic blasts from KMT2A/MLL-R$^+$ AML patients

We examined the PTK gene expression profiles of primary leukemic blasts from 89 children and 38 adults with KMT2A/MLL-R$^+$ AML *vs.* normal hematopoietic cells from healthy volunteers (N = 74). In comparison to normal hematopoietic cells, KMT2A/MLL-R$^+$ pediatric as well as adult AML cells were characterized by amplified expression of FLT3 gene. FLT3_206674_at was the most significantly upregulated probeset (Fold Change in pediatric KMT2A/MLL-R$^+$ AML cells = 7.54; P-value < 1×10^{-8}; Fold Change in adult KMT2A/MLL-R$^+$ AML cells = 8.28; P-value < 1×10^{-8}) followed by TEC_206301_at (Fold Change in pediatric KMT2A/MLL-R$^+$ AML cells = 2.2; P-value < 1×10^{-8}; Fold Change in adult KMT2A/MLL-R$^+$ AML cells = 1.73; P-value < 1×10^{-8}) [Supplementary Figures 4 and 5; Supplementary Tables 7 and 8].

We next compared the PTK gene expression profiles of leukemic cells from pediatric and adult KMT2A/MLL-R$^+$ AML cells to the PTK gene expression profiles of AML cells without KMT2A/MLL rearrangements from the corresponding control AML patients in the "other" categories (Children, N = 190; Adults, N = 504). In pediatric patients with KMT2A/MLL-R$^+$ AML, the gene expression levels for FLT3, BTK, SYK, JAK2/JAK3, as well as several SRC family PTK were differentially amplified [Figure 4; Supplementary Table 9]. In adults with KMT2A/MLL-R$^+$ AML, the gene expression levels for SYK, JAK family kinase TYK2, and the SRC family kinases FGR and HCK were differentially amplified [Figure 5; Supplementary Table 10].

The FLT3-ITD$^+$ subset (N = 48) among the pediatric AML patients exhibited upregulated FLT3 expression when compared to non-leukemic control samples (N = 74) [Supplementary Figure 6; Supplementary Table 11] as well as FLT3-ITD$^-$ pediatric AML cases (N = 189) [Supplementary Figure 7; Supplementary Table 12]. Comparing 189 cases of FLT3-ITD$^-$ samples with 48 cases of FLT3-ITD$^+$ pediatric AML samples (GSE17855) exhibited 13 differentially expressed probesets, of which four probesets were significantly upregulated in pediatric FLT3-ITD$^+$ cases. FLT3_206674_at was the most significantly upregulated probeset (Fold Change = 1.96; P-value < 10^{-8}) followed by BTK_205504_at (Fold Change = 1.25; P-value = 9.8×10^{-4}) and TEC_206301_at (Fold Change = 1.23; P-value = 0.0025) [Supplementary Figure 7; Supplementary Table 12].

A comparison of the 88 KMT2A/MLL-R$^+$ pediatric AML cases with 47 FLT3-ITD$^+$ pediatric AML cases showed 31 differentially expressed probesets, of which 24 probesets were significantly upregulated in pediatric KMT2A/MLL-R$^+$ cases. FGR_208438_s_at was the most significantly upregulated transcript (Fold Change = 4.40; P-value < 10^{-8}) followed by SYK_207540_s_at (Fold Change = 3.81; P-value < 10^{-8}) and JAK1_1552611_a_at (Fold Change = 2.78; P-value < 10^{-8}). ERBB1_1565483_at was the most significantly downregulated transcript in KMT2A/MLL-R$^+$ cases (Fold Change = 0.56; P-value < 10^{-8}) followed by ERBB1_1565484_x_at (Fold Change = 0.57; P-value = 2.5×10^{-8}) and MERTK_211912_at (Fold Change = 0.73; Pval = 0.0016) [Supplementary Figure 8; Supplementary Table 13]

A comparison of the 88 KMT2A/MLL-R$^+$ pediatric AML cases with 71 cases of FLT3-ITD$^+$/NPM1$^+$/CEBPA$^+$ pediatric AML cases exhibited 34 differentially expressed probesets, of which 26 probesets were significantly upregulated in the KMT2A/ MLL-R$^+$ subset. FGR_208438_s_at was the most significantly upregulated probeset (Fold Change = 4.31; P-value < 10^{-8}) followed by SYK_207540_s_at (Fold Change = 3.92; P-value < 10^{-8}) and HCK_208018_s_at (Fold Change = 2.86; P-value < 10^{-8}). ERBB1_1565483_at was the most significantly downregulated transcript in KMT2A/MLL-R$^+$ cases (Fold Change = 0.55; P-value < 10^{-8})

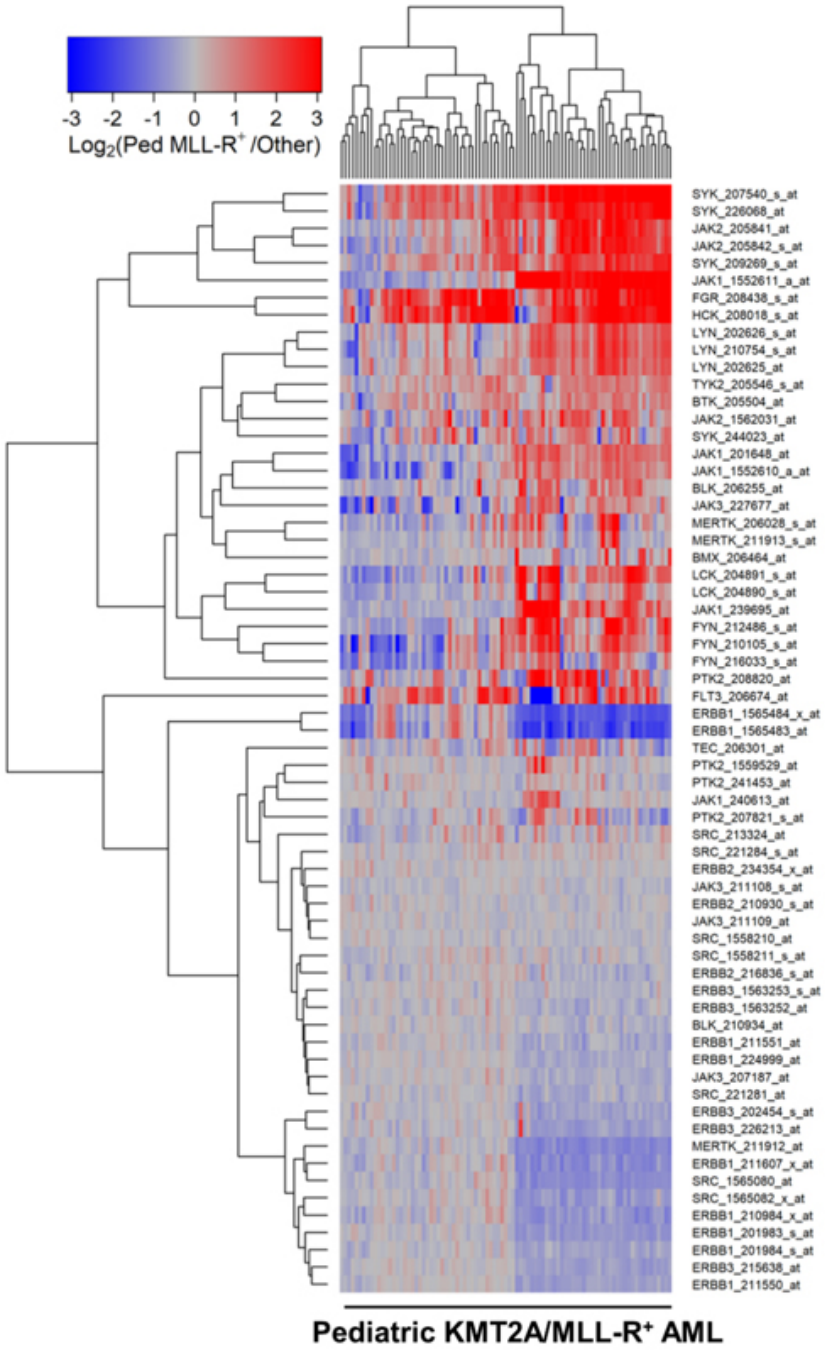

Figure 4. Gene Expression Levels for Tyrosine Kinases in Leukemic Cells from Pediatric Patients with KMT2A/MLL-R$^+$ AML *vs.* Other subsets of AML without KMT2A/MLL rearrangements. Probeset level normalized signal values from the archived data sets GSE17855 and GSE19577 were examined in these comparisons. The cluster figure displays the expression levels in KMT2A/MLL-R$^+$ AML cells mean centered to the reference group (non-KMT2A/MLL-R$^+$ other AML subsets) for $\log_2$-transformed fold change values (blue to red color indicates under-expression to over-expression respectively in KMT2A/MLL-R$^+$ samples). Co-regulated probesets are organized and depicted by dendrograms for both probesets (rows) and patients (columns). Comparing 190 cases of non-KMT2A/MLL-R$^+$ other samples (GSE17855) with 89 cases of KMT2A/MLL-R$^+$ pediatric AML samples [GSE17855 (N = 47) and GSE19577(N = 42)] exhibited 38 differentially expressed probesets, of which 25 probesets were significantly upregulated in pediatric KMT2A/MLL-R$^+$ subset of cases. FGR_208438_s_at was the most significantly upregulated transcript (Fold Change = 4.31 P-value < 10^{-8}) followed by SYK_207540_s_at (Fold Change = 4.01; P-value < 10^{-8}) and HCK_208018_s_at (Fold Change = 3.97; P-value < 10^{-8}) [Supplementary Table 9]. SYK (3 probesets), JAK2 (2 probesets), JAK1, FGR and HCK formed a cluster of patients with significantly higher expression levels in KMT2A/MLL-R$^+$ cases.

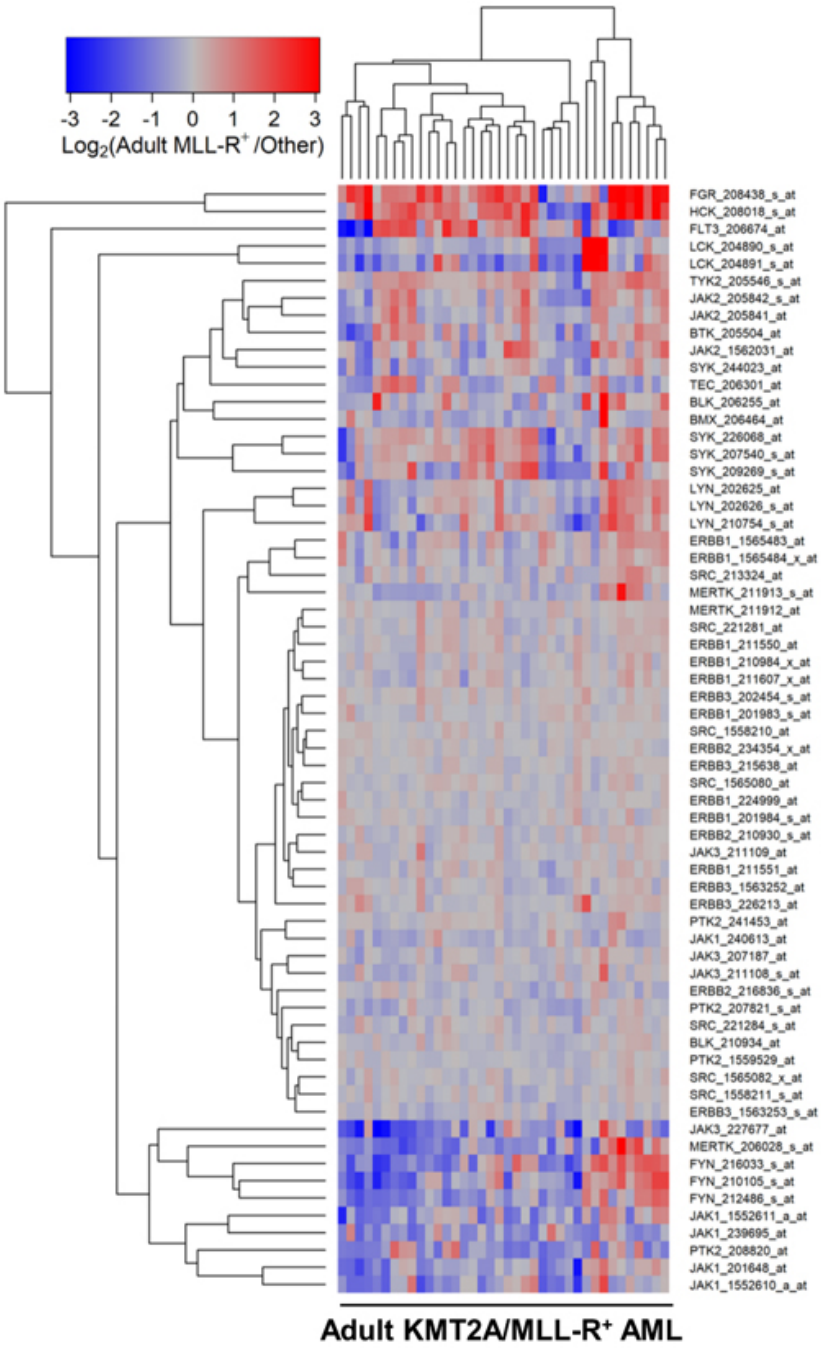

Figure 5. Gene Expression Levels for Tyrosine Kinases in Leukemic Cells from Adult Patients with KMT2A/MLL-R$^+$ AML *vs.* Other subsets of AML without KMT2A/MLL rearrangements. Probeset level normalized signal values from the archived data set GSE13159 were examined in these comparisons. The cluster figure displays the expression levels in KMT2A/MLL-R$^+$ AML cells mean centered to the reference group (other AML subsets without KMT2A/MLL rearrangements) for $\log_2$-transformed fold change values (blue to red color indicates under-expression to over-expression respectively in KMT2A/MLL-R$^+$ samples). Co-regulated probesets are organized and depicted by dendrograms for both probesets (rows) and patients (columns). Side-by-side comparison of 38 adult MLL-R$^+$ AML cases with 504 other non-MLL-R$^+$ adult AML cases resulted in 15 dysregulated probesets, of which 6 were upregulated in the adult KMT2A/MLL-R$^+$ subset of cases. FGR_208438_s_at was the most significantly upregulated probeset (Fold Change = 2.46; *P*-value < 10^{-8}) followed by HCK_208018_s_at (Fold Change = 2.1; *P*-value < 10^{-8}) and TYK2_205546_s_at (Fold Change = 1.29; *P*-value = 0.0012) [Supplementary Table 10]. Cluster visualization of the mean centered expression values suggested co-regulation of CD33 with BTK, TYK2 and SYK (3 probesets).

followed by ERBB1_1565484_x_at (Fold Change = 0.56; *P*-value < 10^{-8}) and MERTK_211912_at (Fold Change = 0.72; *P*-value = 1.5×10^{-4}) [Supplementary Figure 9; Supplementary Table 14].

DISCUSSION

The outcome of KMT2A/MLL-R$^+$ AML and B-ALL patients, especially those with relapsed or refractory leukemia, is disappointingly poor after contemporary treatments[3,5-8,10-14]. New treatment strategies are urgently needed for both KMT2A/MLL-R$^+$ AML and KMT2A/MLL-R$^+$ ALL. Therefore, a large panel of drugs are being evaluated as potential therapeutic agents against KMT2A/MLL-R$^+$ AML and ALL, including inhibitors of Menin-MLL1 interaction[25-28]. The present study demonstrates that PTK inhibitors (PTKi), especially inhibitors of FLT3, may have clinical impact potential as therapeutic agents against KMT2A/MLL-R$^+$ B-ALL as well as AML. Further, inhibitors of SRC family PTK may be clinically useful against KMT2A/MLL-R$^+$ B-ALL and inhibitors of BTK, SYK, and JAK family PTK may be clinically useful against KMT2A/MLL-R$^+$ AML. If our observations in this small series are confirmed in a larger series of leukemia patients, preferably with corresponding proteomics/phosphoproteomics data, proof-of-concept studies with FDA-approved PTKi would be warranted to further evaluate the clinical potential of PTK targeting in KMT2A/MLL-R$^+$ ALL and AML.

PTK play a critical role in normal lymphohematopoiesis, and they have also been implicated as leukemogenic oncoproteins in the development of acute and chronic leukemias[29-31]. The incorporation of PTKi into the standard of care has caused a paradigm shift in the treatment of CML (PTKi: ABL1 and SRC inhibitors), CLL (PTKi: BTK inhibitors), Ph$^+$ B-ALL (PTKi: ABL1 and SRC inhibitors), and AML with *FLT3* mutations (PTKi: FLT3 inhibitors)[29,30]. There is growing consensus regarding their evolving role in Ph-like B-ALL (PTKi: ABL1, SRC, TRK, FLT3 and JAK inhibitors) and pre-B ALL with t(1;19) (PTKi: BTK and SRC inhibitors)[30-53] [Table 1]. Notably, Ph$^+$ B-ALL was associated with a very poor outcome in both children and adults, with less than 20% long-term survival until the introduction of PTKi capable of inhibiting the oncogenic BCR-ABL tyrosine kinase[36-48] [Table 1]. Due to very high rates of deep complete remissions and markedly improved long-term EFS and OS achieved with combinations of PTKi and standard chemo- or biotherapy, patients with Ph$^+$ B-ALL are no longer considered to have a poor prognosis. The standard of care is being optimized by developing novel combinations of PTKi such as dasatinib and ponatinib with bispecific antibodies such as blinatumomab, CAR-T cells, and antibody-drug conjugates and using less toxic chemotherapy regimens[36-48]. Likewise, FLT3 inhibitors have contributed to improved outcomes in AML patients with a *FLT3* mutation[49]. FLT3 is a receptor tyrosine kinase that plays an important role in normal lymphohematopoiesis[50]. Leukemic cells from AML patients abundantly express FLT3 and many AML patients have activating mutations of FLT3, including internal tandem duplication mutations (FLT3-ITD) and kinase domain activation loop mutations (FLT3-ALM)[50,51]. *FLT3* mutations are rare in ALL, but a high-level expression of FLT3 was also reported in MLL-AF4 positive ALL patients and showed poor prognostic value[52,53]. Our study significantly expands the knowledge regarding FLT3 expression in ALL as well as AML by demonstrating amplified expression levels in infants, children, and adults with KMT2A/MLL-R$^+$ B-ALL and children with KMT2A/MLL-R$^+$ AML. Several PTKi have been approved for the treatment of AML with *FLT3* mutations, including midostaurin and gilteritinib [Table 1]. Our results suggest that FLT3 inhibitors may have clinical potential as therapeutic agents against KMT2A/MLL-R$^+$ ALL. It is noteworthy that Dovitinib, a multi-functional PTKI with FLT3 inhibitory activity, was reported to exhibit nanomolar in vitro activity against KMT2A/MLL-R$^+$ ALL cells[54].

One of the intriguing findings of our study relates to the amplified expression of the gene for FLT3 in KMT2A/MLL-R$^+$ pediatric AML cells. Except for GSE17855, the archived datasets used in the present study did not contain information about the FLT3 status of the respective leukemia cases. Additional

Table 1. FDA-approved Inhibitors of FLT3, ABL1, SRC, BTK/TEC, KIT, SYK, and JAK1-3

Target kinase	Drug	Brand
ABL1, SRC	Bosutinib	Bosulif, SKI-606
ABL1, SRC, FGR, CKIT	Dasatinib	BMS- 354825, Sprycell
ABL1, CKIT, PDGFR	Imatinib	STI571, Gleevec
ABL1	Nilotinib	AMN107, Tasigna
ABL1	Olverembatinib	HQP1351
ABL1, SRC, FGR	Ponatinib	Iclusig
BTK/TEC	Ibrutinib	PCI-32765, Imbruvica
BTK	Zanubrutinib	BGB3111, Brukinsa
BTK	Acalabrutinib	Calquence
FLT3	Midostaurin	CPG 41251, Rydapt
FLT3	Gilteritinib	ASP2215, Xospata
JAK1	Upadacitinib	ABT-494, Rinvoq
JAK1/2	Baricitinib	Olumiant, LY 3009104
JAK1/2/3, Tyk	Ruxolitinib	Jakafi
JAK2	Fedratinib	TG101348, Inrebic
JAK3	Tofacitinib	Xeljanz
KIT/PDGFR	Ripretinib	DCC- 2618, Qinlock
SYK	Fostamatinib	R788, Tavalisse
TRKA/B/C	Larotrectinib	LOXO-101, Vitrakvi
TRKA/B/C, ROS1	Entrectinib	RXDX-101, Ignyta, Rozlytrek
VEGFR2, FLT3, CKIT	Sunitinib	Sutent

KMT2A/MLL-R$^+$ patients whose leukemia cells do not have amplified FLT3 gene expression but an activating *FLT3* mutation like FLT3-ITD may also benefit from the use of FLT3 inhibitors. Analysis of the GSE17855 contributed to our finding that in pediatric patients with KMT2A/MLL-R$^+$ AML, the gene expression level for FLT3 is differentially amplified. It is noteworthy that only one patient with KMT2A/MLL-R$^+$ AML had FLT3-ITD whereas 47 patients from the KMT2A/MLL-R negative "other" group of pediatric AML patients were FLT3-ITD$^+$, including patients with cytogenetically normal AML (N = 18); AML with inv[16] (N = 1); AML with t(15;17) (N = 12); AML with t(8;21) (N = 3); AML with other cytogenetic features (N = 9); and AML with unknown cytogenetic features (N = 4). Therefore, our results should not be interpreted to suggest that FLT3 inhibitors would be preferentially active in pediatric AML patients with KMT2A/MLL rearrangements, as many cases without KMT2A/MLL rearrangements whose leukemia cells are FLT3-ITD$^+$ may also benefit from FLT3 inhibitors. Some PTK, such as FLT3, participate in immune suppression mediated by leukemia cells, which may promote the immune escape of leukemic clones[55]. Therefore, their inhibition with PTKi may partially contribute to favorable treatment outcomes by lifting the immune suppression. For example, the PTKi sorafenib has been shown to abrogate the transcriptional downregulation of interferon regulatory factor 7 (IRF7), which resulted in an augmented CD8$^+$CD107a$^+$IFN-γ^+ T cell response in mouse models of FLT-ITD$^+$ AML[55]. Mathew *et al.* proposed that sorafenib may therefore exhibit immune-mediated anti-leukemic activity in FLT3-ITD mutant AML[55].

The SRC kinase family includes several cytoplasmic PTK, including BLK, HCK, FGR, LYN, FYN, and LCK, which have important regulatory functions for signal transduction pathways related to survival, proliferation, and apoptosis of leukemic cells[29-31]. FDA-approved SRC kinase inhibitors have become part of the standard of care in the treatment of CML as well as Ph$^+$ ALL[36-48] [Table 1]. In the current study, we discovered the amplified expression of the genes for BLK, HCK, FGR as well as LYN in primary leukemic cells from infants, children as well as adults with KMT2A/MLL-R$^+$ B-ALL. The gene for BLK was expressed

at a 6.77-fold higher level in infant KMT2A/MLL-R$^+$ ALL cells, 4.38-fold higher level in pediatric KMT2A/MLL-R$^+$ ALL cells, and 21.79-fold higher level in adult KMT2A/MLL-R$^+$ ALL cells than in normal hematopoietic cells. Notably, the expression of the genes for BLK, HCK and LYN in primary leukemic cells from KMT2A/MLL-R$^+$ ALL patients was differentially and significantly amplified regardless of the age group compared to the expression levels in leukemic cells from B-ALL patients without KMT2A/MLL gene rearrangements. Likewise, the genes of several SRC family PTK were differentially upregulated in pediatric and adult KMT2A/MLL-R$^+$ AML cells. The availability of FDA-approved potent inhibitors of SRC family PTK such as ponatinib, bosutinib, and dasatinib [Table 1] provides an opportunity to evaluate their clinical impact potential for KMT2A/MLL-R$^+$ leukemias in proof-of-concept clinical trials. The insights and lessons learned in the clinical development of these PTKi as precision medicines against Ph$^+$ ALL should facilitate their development as potential precision medicines against KMT2A/MLL-R$^+$ ALL as well.

Additional insights from the present study relate to the upregulation of SYK and JAK expression in pediatric and adult KMT2A/MLL-R$^+$ acute leukemias. These results extend our earlier studies regarding upregulation of the JAK-STAT pathways in infant B-ALL[56,57] and upregulation of SYK expression in pediatric B-ALL[58-62]. The availability of FDA-approved potent inhibitors of SYK and JAK provides the opportunity to evaluate their clinical efficacy in KMT2A/MLL-R$^+$ acute leukemias with overexpression of their respective targets. A significant portion of high-risk acute leukemia patients, especially those with KMT2A/MLL-R$^+$ ALL or AML relapse after being treated on contemporary chemotherapy protocols due to cancer drug resistance of their leukemic clones, and the survival outcome of available salvage regimens is disappointing due to the short nature and poor quality of second remissions. We previously reported that the JAK/STAT signaling pathway is constitutively active in infant pro-B ALL cells and treatment with a JAK3 inhibitor or a pan-JAK kinase inhibitor effectively triggered their apoptosis[56,57]. JAK targeting with a small molecule inhibitor may be a viable strategy to overcome cancer drug resistance in KMT2A/MLL-R$^+$ B-ALL cells. Constitutively active JAK2-STAT5 signaling has been shown to be associated with increased surface PD-L1 expression due to amplified PD-L1 promoter activity in myeloproliferative neoplasms, which may facilitate PD-L1-mediated immune escape[63,64]. JAK2 inhibition reduces the PD-L1 expression levels and may therefore mitigate this risk.

SYK has been discovered to regulate the cancer drug resistance-related anti-apoptotic STAT3, NF-κB as well as PI-3K-AKT-mTOR pathways[60-62]. Our earlier studies have identified high-level SYK expression as a likely contributor to cancer drug resistance and relapse in B-ALL[60,61]. Upregulation of SYK expression was associated with significant upregulation of at least one of the STAT3 target genes[60]. Inhibition of SYK caused apoptotic death in primary leukemia cells from B-ALL patients that are resistant to chemotherapy[61]. Notably, a nanomedicine candidate containing the SYK-inhibiting small molecule compound 1,4-bis(9-O-dihydroquinidyl) phthalazine/hydroquinidine1,4-phathalazinediyldiether (C61) was capable of destroying > 99.9% of clonogenic B-ALL cells in vivo and thereby improved the event-free survival outcome of SCID mice challenged with otherwise invariably fatal doses of human leukemic B-cell precursors in each of three different xenograft models of chemotherapy-resistant human B-ALL[62]. Taken together with these earlier observations, the amplified expression of SYK in the poor prognosis KMT2A/MLL-R$^+$ ALL patients prompts the hypothesis that SYKi may help overcome the cancer drug resistance of relapse clones and thereby provide the foundation for more effective multi-modality treatment regimens for KMT2A/MLL-R$^+$ acute leukemias.

DECLARATIONS

Authors' contributions

Designed the evaluations reported in this paper, directed the data compilation and analysis, and prepared the initial draft of the manuscript: Uckun FM

Performed statistical and bioinformatic analyses: Qazi S
Analyzed, validated data and reviewed and revised the manuscript, and provided final approval for submission of the final version: Uckun FM, Qazi S

Availability of data and materials

All data are presented in the manuscript and the Supplemental Material. The publicly available archived databases that were used to generate the data have been provided in Materials and Methods. Specifically, raw Affymetrix .CEL data files on gene expression profiles of AML cells analyzed in the current study were obtained from 3 publicly available datasets deposited in the NCBI repository (GSE13159, GSE19577, and GSE17855). Raw Affymetrix .CEL data files on gene expression profiles of ALL cells analyzed in the current study were obtained from 8 publicly available datasets deposited in the NCBI repository (GSE11877, GSE13159, GSE13351, GSE18497, GSE28460, GSE7440, GSE68720, and GSE32962).

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

IRB approval was not required as the analysis of de-identified, publicly available data does not constitute human subjects research as defined at 45 CFR 46.102.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Perspective

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Tackling heterogeneity in treatment-resistant breast cancer using a broad-spectrum therapeutic approach

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Abstract

Tumor heterogeneity can contribute to the development of therapeutic resistance in cancer, including advanced breast cancers. The object of the Halifax project was to identify new treatments that would address mechanisms of therapeutic resistance through tumor heterogeneity by uncovering combinations of therapeutics that could target the hallmarks of cancer rather than focusing on individual gene products. A taskforce of 180 cancer researchers, used molecular profiling to highlight key targets responsible for each of the hallmarks of cancer and then find existing therapeutic agents that could be used to reach those targets with limited toxicity. In many cases, natural health products and re-purposed pharmaceuticals were identified as potential agents. Hence, by combining the molecular profiling of tumors with therapeutics that target the hallmark features of cancer, the heterogeneity of advanced-stage breast cancers can be addressed.

Keywords: Breast cancer, chemoresistance, drug resistance, targeted therapy

Breast cancer is a consequence of complex epigenetic and genetic alterations. The heterogeneity and

evolution within breast cancers underpin tumor progression, as well as therapeutic resistance[1-3]. In recent years, significant efforts have focused on addressing therapeutic options that can address tumor heterogeneity in breast cancer[4-6]. This is particularly important in triple-negative breast cancers and metastatic breast cancers because many patients with advanced breast cancers will succumb to their disease as tumor heterogeneity gives rise to therapeutic resistance[7-9] [Figure 1].

To address the challenge of therapeutic resistance and tumor heterogeneity, a group of 180 cancer researchers collaborated in "The Halifax Project" to consider combinations of agents that might be employed[10]. In this effort, twelve teams of researchers were organized around the Hallmarks of Cancer[11] and tasked to identify high-priority targets along with corresponding therapeutic agents that could reach those targets with limited toxicity. The hallmarks used are attributes ultimately found in most cancers (i.e., genomic instability, sustained proliferative signaling, tumor-promoting inflammation, evasion of anti-growth signaling, resistance to apoptosis, replicative immortality, dysregulated metabolism, immune system evasion, angiogenesis, tissue invasion and metastasis, and an accommodating tumor micro-environment). The overarching goal was to identify a significant number of agents that have limited to no toxicity, that might be combined to reach a multitude of key targets simultaneously.

Cancer is caused by an array of mutations and genomic events that coordinate to activate aberrant pathways. To address this complexity, we used the Hallmarks of Cancer as an organizing framework. We then focused on the development of a "broad-spectrum" methodology and therapeutic agents that could be combined using this approach. In particular, we focused on identifying natural health products (NHPs) and re-purposed pharmaceuticals, because both are readily available, often well tolerated, and broadly applicable to many cancers[10,12-22]. A detailed rationale for the methodology was provided and it was determined that it should be feasible from a safety standpoint and relatively inexpensive to implement[23].

In the table below [Table 1] we have provided a sampling of NHP with updated references to show how these agents act on key mechanisms and pathways across the hallmarks of cancer[10-22].

Some NHPs, such as curcumin and resveratrol, target multiple signaling networks and pathways simultaneously which is attractive molecular promiscuity[68,69]. The appropriate selection of NHPs can also offer synergies for chemotherapy and radiation therapy treatment. For example, curcumin, resveratrol, tocotrienol, garcinol and quercetin have a mechanism of action that increases chemosensitivity[70,71] and can reduce chemoresistance. While other NHPs, such as ellagic acid, diindolylmethane, and berberine, can increase radiation sensitivity[72-74].

Other NHPs, such as gingerol and curcumin can also protect normal (healthy) cells from adverse toxicity from cytotoxic agents[75,76]. Thus, NHPs have many features that may designate them particularly suited as agents that might be used in situations where tumor heterogeneity has resulted in chemoresistance.

In particular, for breast cancer, the presence of subpopulations of cancer stem cells (CSCs) is known to be one cause of chemo-resistance and ultimately contributes to therapeutic relapse[77]. Notably, there are specific NHPs such as sulforaphane, curcumin, genistein, resveratrol, lycopene, and epigallocatechin-3-gallate that have been shown to promote cell cycle arrest and apoptosis in triple-negative breast cancer cells and which have also been shown to inhibit important CSC pathways, such as NF-κB, PI3K/Akt/mTOR, Notch 1, Wnt/β-catenin, and YAP[78].

Table 1. Aligning targets with the hallmarks of cancer

Cancer hallmark	Examples of potential agents	Key mechanism or pathway
Genomic instability	Allyl Isothiocyanate[24] Chrysin[25] Plumbagin[26]	DNA damage and condensation DNA double-strand break repair DNA damage
Sustained proliferative signaling	Resveratrol[27] Perillyl alcohol[28] Artemisinin[29]	Cell cycle Cell cycle Cell cycle
Tumor-promoting Inflammation	Rosmarinic acid[30] Berberine[31] Curcumin[32] Punica granatum L[33]	NF3 kappaB-p53-caspase-3 pathways NLRP3 Inflammasome pathway Nuclear factor-κB (NF-κB) miRNA-27a and miRNA-155
Evasion of anti-growth signaling	Deguelin[34] Luteolin[35] Withaferin A[36,37] Curcumin[38]	EGFR-p-AKT/c-Met p-ERK AKT/mTOR pathway Notch2 SLC1A5-mediated ferroptosis
Resistance to apoptosis	EGCG[39] Gossypol[40] Triptolide[41] Kaempferol[42] Berberine[43,44]	P53/Bcl-2 pathway miRNA expression of many apoptosis related genes p38/Erk/mTOR Bcl2 Bcl2 (and many other pathways)
Replicative immortality	Curcumin[45,46] Silibinin[46] Coumestrol[47] Diosmin[48]	Telomerase expression Telomerase expression Protein kinase CKII Senescence
Dysregulated metabolism	Resveratrol[49] Metformin[50] Baicalein[51] Carpesium abrotanoides L.[52]	6-phosphofructo-1-kinase HIF-1alpha HIF-1alpha Glucose Metabolism and PKM2/HIF-1alpha axis
Immune system evasion	Astragalus polysaccharides[53] Cordycepin[54] Resveratrol[55]	Macrophage activation IL-2, TGF-β, IL-4 MICA/B and natural killer cells
Angiogenesis	Curcumin[56] EGCG[57] Melatonin[58,59] Resveratrol[60]	NF-κB pathway VEGF VEGF VEGF
Tissue invasion and metastasis	Diallyl trisulfides[61] Resveratrol[62] Anthocyanins[63] Cordycepin[64]	HIF-1alpha TGF-beta1 / Epithelial-Mesenchymal Transition FAK Hedgehog pathway
Tumor micro-environment	Resveratrol[65] Sulforaphane[66,67]	Macrophage polarization Adipose mesenchymal stem cells

FAK: Focal adhesion kinase; EGCG: epigallocatechin gallate; VEGF: vascular endothelial growth factor.

In addition to NHPs, there are also many existing pharmaceuticals that could provide additional targeting options. Numerous commonly prescribed non-oncology drugs possess multi-targeted anti-cancer effects. Pharmaceuticals already on the market have significant safety records and robust drug-drug interaction data compared to natural products and several researchers have looked at the effects of existing pharmaceuticals as it relates to relapse. Retsky (2012, 2020), for example, observed that the perioperative use of the NSAID analgesic ketorolac appears to reduce early relapse following mastectomy in breast cancer[79,80]. Hence, the use of both NHPs and repurposed pharmaceuticals to reach a broad-spectrum of molecular targets could be useful in developing personalized treatment protocols[81] [Figure 2].

Although our proposed approach has many potential advantages, there are challenges to conducting validating clinical studies. First, there is a shortage of funding for this type of initiative due to lack of patentability, manufacturing difficulties, contamination, and lack of product consistency[82]. Second, the use of NHPs among cancer patients is quite common. In fact, many patients who use them do not share the details with their physicians because they feel their physicians are not knowledgeable or will be indifferent

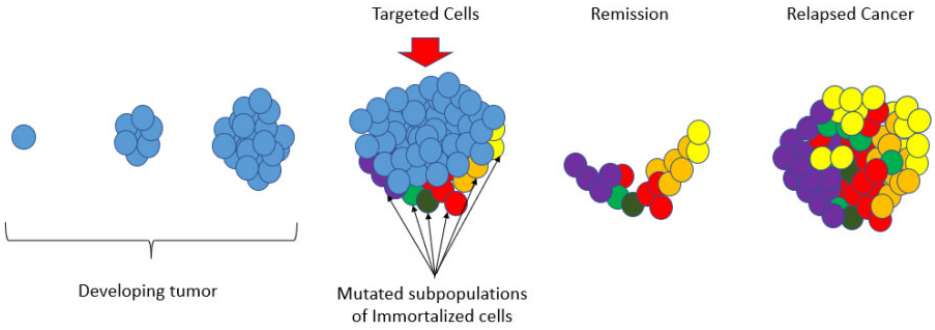

Figure 1. Therapeutic resistance - Developing tumors begin with a single immortalized cell that may have a single therapeutic target that can act to stop those cells from replication. For example, Tamoxifen (TAM) is the most common therapy used for the treatment of estrogen receptor-positive (ER+) breast cancer and it is used successfully in many cases. However, there are advanced-stage breast cancers that are plagued with mutated subpopulations of immortalized cells and cancer stem cells that play a key role in breast cancer progression, and metastasis. In these cancers, a single targeted therapy may produce a remission by successfully arresting some of the immortalized cells that have been targeted. However, if the remaining subpopulations of cells are driven by different mechanisms and prove to be chemoresistant, they will persist during remission and ultimately produce a relapsed cancer that is fully refractory to the initial treatment or combination of treatments.

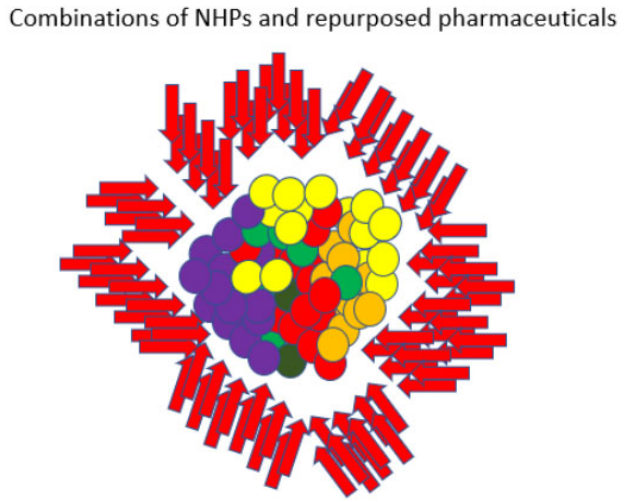

Figure 2. A broad-spectrum approach - In a broad-spectrum approach, heterogeneous subpopulations of chemoresistant immortalized cells are not targeted using a single targeted therapy or even a combination of 2-3 chemotherapy agents. Instead, a significant number of low toxicity agents are aimed at a multitude of key pathways/mechanisms simultaneously. Since most immortalized cells are driven by the pathways/mechanisms described in the Hallmarks of Cancer framework[11], this approach increases the chance that a significant number of synergistic effects will be produced (i.e., since each affected cell will potentially be acted on in a multitude of ways).

or negative toward their use[83,84]. Finally, NHPs have yet to be approved by the FDA and although many NHPs are available as supplements and generally well-tolerated over an extended duration, the clinical evidence for these agents is often weak or non-existent.

One example of how NHPs can be used to improve the treatment of breast cancer is that NHPs have been shown to combine with Tamoxifen synergistically in inhibition of tumor cell growth, improved Tamoxifen sensitivity and reduction of Tamoxifen side effects[85]. However, some NHPs showed estrogen-like activity, which could reduce the effect of Tamoxifen, underscoring the need for a detailed analysis of any protocol that combines a multitude of agents[85]. They did find that some NHPs (e.g., morin, silybin, epigallocatechin gallate, myricetin, baicalein, curcumin, kaempferol, and quercetin) helped to increase the bioavailability of

Tamoxifen *in vivo*. These promising observations suggest that NHPs with Tamoxifen are worthy of clinical studies.

Some NHPs are used to support cancer therapy by clinicians who practice integrative oncology; however, these physicians are typically less familiar with the molecular mechanisms of cancer signaling[86]. Instead, integrative oncology mainly focuses on the treatment of cancer-related symptoms such as acupuncture for nausea, exercise for sleep, and anxiety[86]. Indeed, a survey of clinics in Washington State showed that more than 72 oral or topical, nutritional, botanical, fungal and bacterial-based medicines had been used during the first year of care of the female breast cancer patients studied (n = 324)[87]. Since most of these agents are not aimed at the molecular mechanisms of cancer, the use of NHPs for these purposes would typically not include the type of analysis that would be needed to target tumor heterogeneity.

We highlight this approach as an important avenue that should be investigated further because the idea of reaching many key targets simultaneously makes sense given what is now known about the biology of cancer. Importantly, this is not something that has been attempted previously. Clinical trials of NHPs that have been undertaken typically involve single agents or limited combinations of agents at best. We speculate the use of combinations of NHPs that are able to hit multiple targets is most likely to be clinically effective.

The goal should be to provide a clinical treatment protocol that makes a rational utilization of the evidence base. If this approach is to work, future efforts utilizing NHPs and repurposed pharmaceuticals will require clinical studies involving unique combinations of dozens of agents in protocols that are tailored/personalized for each patient. The agents that are used will therefore need to be carefully considered for potential interactions, and some of these agents may have shown limited or no activity when used individually. Until there is clinical research that fully explores the synergies that can be produced when a significant number of pathways are targeted simultaneously, the true potential of combining these actions all at once will simply not be known.

Finally, we acknowledge that what we are proposing would require a change from phased clinical trials. A case series or a cohort study might be a more appropriate means to document the results of experimental efforts of this nature[88], since each patient will require an individualized protocol. We do believe that such an approach may be able to help address the challenges of therapeutic resistance that emerge in many breast cancers.

DECLARATIONS

Authors' contributions

All authors contributed equally.

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Original Article

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Discovery of the inhibitor of DNA binding 1 as a novel marker for radioresistance in pancreatic cancer using genome-wide RNA-seq

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Abstract

Purpose/Objective(s): Discovery of genetic drivers of radioresistance is critical for developing novel therapeutic strategies to combine with radiotherapy of radioresistant PDAC. In this study, we used genome-wide RNA-seq to identify genes upregulated in generated radioresistant PDAC cell lines and discovered the Inhibitor of DNA Binding 1 (ID1) gene as a potential regulator of radioresistance in PDAC.

Materials/Methods: Radioresistant clones of the PDAC cell lines MIA PaCa-2 and PANC-1 were generated by delivering daily ionizing irradiation (IR) (2 Gy/day) *in vitro* over two weeks (total 20 Gy) followed by standard clonogenic assays following one week from the end of IR. The generated RR and parental cell lines were submitted for RNA-seq analysis to identify differentially expressed genes. The Limma R package was used to calculate differential expression among genes. Log2 fold change values were calculated for each sample compared to the control. Genes with an absolute fold change > 1 were considered significant. RNA sequencing expression data from the Cancer Genome Atlas (TCGA) database was analyzed through the online databases GEPIA, cBioPortal, and the Human Protein Atlas.

Results: Following exposure to two weeks of 2 Gy daily IR in vitro, the two PDAC cell lines showed significantly greater clonogenic cell survival than their parental cell lines, indicating enhanced RR in these cells. RNA-seq analysis comparing parental and RR cell lines found upregulated seven genes (TNS4, ZDHHC8P1, APLNR, AQP3, SPP1, ID1, ID2) and seven genes downregulated (PTX3, ITGB2, EPS8L1, ALDH1L2, KCNT2, ARHGAP9, IFI16) in both RR cell lines. Western blotting confirmed increased expression of the ID1 protein in the RR cell lines compared to their parental cell lines. We found that ID1 mRNA was significantly higher in PDAC tumors compared to matched normal and high ID1 expression correlated with significantly worse disease-free survival (DFS) in PDAC patients (HR = 2.2, log rank P = 0.009). ID1 mRNA expression was also strongly correlated in tumors with TP53 mutation, a known driver of radioresistance.

Conclusion: Our analysis indicates a novel role of ID1 in PDAC radioresistance. ID1 expression is higher in tumor tissue compared to normal, and high expression correlates with both worse DFS and association with the TP53 mutation, suggesting that targeting ID1 prior to IR is an attractive strategy for overcoming radioresistance in PDAC.

Keywords: Pancreatic cancer, radiation resistance, ID1, RNA-seq

INTRODUCTION

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most lethal solid tumors with a rapid progression course and poor prognosis, contributing to a 10% of five-year survival rate for all stages[1]. By the year 2040, the incidence of PDAC is expected to increase from 56,000 to 96,000 cases, a 66% increase. Pancreatic cancer is projected to become the second leading cause of cancer-related death, killing ~46,000 patients yearly in the U.S. alone[2]. The only hope for long-term survival in patients with localized PDAC is surgical resection of the tumor. However, upwards of 60% of the patients with potentially curable localized PDAC do not present with frank eligibility for surgical resection[3], and therefore require an aggressive therapy regimen to reduce tumor burden enough to elicit removal. The standard course includes 3-6 months of multi-regimen genotoxic chemotherapies and, commonly, an additional 3-6 weeks of radiation therapy (RT). For most locally advanced PDAC patients, curative surgery will not be achieved despite these aggressive therapies due to the poor response of the tumor and thus remain unresectable. The rate of conversion from unresectable to resectable disease remains at a dismal ~15%[4]. Unresected patients have nearly the same dismal survival rates as metastatic disease, with a prognosis of roughly 15-16 months and a poor quality of life[5]. Why PDAC responds so poorly to typically effective strategies in other cancers is not entirely clear. It is speculated that the natural prevalence of robust DNA repair mechanisms in PDAC underlies resistance to genotoxic therapy[6]. Therefore, understanding the biological underpinnings of radioresistant systems in PDAC is critical for uncovering vulnerabilities in the resistant phenotype.

In this study, we generated radiation-resistant (RR) PDAC cell lines *in vitro* by treating them with daily doses of ionizing radiation and allowing the cells to recover, followed by an analysis of the changes in gene expression. Through next-generation RNA sequencing (RNA-seq), we discovered several novel genes differentially expressed after RR, including the inhibitor of DNA binding-1 (ID1). The ID1 gene has been previously shown to be upregulated in pancreatic cancer cells following nicotine stimulation via a Src kinase-dependent fashion leading to chemoresistance to gemcitabine treatment. Furthermore, elevated ID1 was shown to correlate with worse overall survival in resected pancreatic cancer patients[7]. Another study in pancreatic cancer showed ID1 uncouples TGFβ-induced EMT from apoptosis leading to enhanced cell survival[8]. The current study validates ID1 as a marker for radiation resistance in PDAC cells for the first time.

METHODS

Cell culture and materials

The human pancreatic adenocarcinoma cell lines MIA PaCa-2 and PANC1 were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37 °C in 5% CO_2 in DMEM media and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and penicillin/streptomycin (Life Technologies, Grand Island, NY).

Generation of radioresistant PDAC cell lines

Cells were subjected to 2 Gray (Gy) radiation daily for 5 days, and then allowed to recover for two weeks, followed by a second course of radiation of 2 Gy daily for 5 days for a total of 20 Gy. Throughout the irradiation process and recovery time, cells were kept at 40%-70% confluency to ensure the potential for exponential growth. After completion of the second week of radiation, cells were collected for protein lysates, plated as single cells for clonogenic assays, or submitted for whole genome sequencing. Radioresistance of the cell lines was verified by comparing the radiosensitivity of the radiation-selected cells (after a recovery period) with their respective parental cell lines by the clonogenic assay as described below.

Immunoblotting

Cell lysates were prepared using RIPA buffer (ThermoFisher, Waltham MA) supplemented with 1x protease (Complete, Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP, Roche, Indianapolis, IN, Roche), followed by protein quantification by the Dc protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded and resolved by SDS/PAGE and transferred to nitrocellulose membranes. The ID1 (Santa Cruz sc-133104) and GAPDH (Cell Signaling D16H11) primary antibodies were allowed to bind overnight at 4 °C and used at a dilution of 1:100-1000. After washing in TBS-Tween, membranes were incubated with StarBright Goat Anti-Mouse/Rabbit IgG secondary antibodies (Bio-Rad) diluted 1:2500-1:5000 for 1 h. Membranes were washed with TBS-Tween and then imaged on the ChemiDoc MP Imaging System (Bio-Rad).

Radiation clonogenic assays

Cells were trypsinized to generate single cell suspensions and seeded onto 60 mm tissue culture plates in triplicate. Cells were then irradiated with various doses (0-8 Gy). Ten to 14 days after seeding, colonies were fixed with Methanol/Acetic Acid and stained with 0.5% crystal violet, and the numbers of colonies or colony forming units (CFU) containing at least 50 cells were counted using a dissecting microscope (Leica Microsystems, Inc. Buffalo Grove, IL) and surviving fractions calculated. Experiments were repeated multiple, independent times.

Experimental radiation

Irradiation was performed with the X-ray at 160 kV, 25 mA at a dose rate of approximately 113 cGy/min using an X-RAD 320 Biological Irradiator (Precision X-Ray Inc.).

RNA library construction and sequencing

Total RNA was isolated with the TRIzol Reagent protocol (ThermoFisher, Waltham, MA) from the parental and RR cell lines. RNA quality and concentration were estimated with Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). RNA-Seq libraries were prepared with Illumina TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA) using 1 μg of total RNA according to the manufacturer's protocol. Sequencing was performed by the genomics core at UAMS on the NovaSeq 6000 platform (Illumina).

RNA-seq analysis

RNA-seq reads were quality-checked, trimmed, and aligned to the hg38 reference genome (accession: GCA_000001405.15) using the Nextflow RNAseq pipeline, nf-core/rnaseq (version 3.4) available at DOI 10.5281/zenodo.1400710. The resulting gene counts were transformed to log2 counts per million (CPM)[9]. Lowly expressed genes were filtered out and libraries were normalized by the trimmed mean of M-values[10]. The Limma R package was used to calculate differential expression among genes[11]. Log2 fold change values were calculated for each sample compared to the control. Genes with an absolute fold change > 1 were considered significant.

Human patient data

The following websites were utilized to query for tumor and normal expression and clinical data for patients with PDAC in the TCGA: https://cbioportal.org/[12], http://gepia.cancer-pku.cn/[13], and https://www.proteinatlas.org/[14].

Statistical analysis

For *in vitro* experiments, data are presented as the mean ± SEM for clonogenic survival. Statistical comparisons were made between the control and experimental conditions using the two-sided two-group *t*-tests with significance assessed at $P < 0.05$.

RESULTS

Generation of radioresistant cell lines

To generate RR cell lines, we delivered 10 Gy of IR (2 Gy/day) to PDAC MIA PACA-2 and PANC-1 cells *in vitro* over five days, followed by a recovery time of 2 weeks followed by a second course of 10 Gy in 2 Gy per day fractions followed by an additional two weeks of recovery [Figure 1]. In parallel, we cultured the parental cells and exposed them to 10 daily doses of sham radiation. Following the generation of RR cells, we performed radiation clonogenic assays comparing the parental and RR cells. In addition, we performed whole-genome sequencing on the parental controls and RR cells in triplicate using the next-generation NovaSeq 6000 RNA sequencer.

In both the PDAC cell lines, the generated RR cells showed increased clonogenic survival after exposure to increasing doses of radiation *in vitro* [Figure 2]. For example, at doses of 4 and 6 Gy, the RR cells displayed roughly 50% more clonogenic survival than the parental controls. These clonogenic assay results confirm the increased RR potential of our newly generated PDAC cell lines.

RNA-seq reveals changes in gene expression following RR

RNA-seq analysis comparing global expression levels in the RR cells to parental cells revealed notable differential expression profiles. In the MIA PACA-2 cell line, there was a total of 65 genes found to be upregulated in the RR cells and 136 genes downregulated (adj $P < 0.05$) [Figure 3A]. In the PANC-1 cells, there were a total of 222 genes upregulated in the RR cells and 156 genes downregulated (adj $P < 0.05$) [Figure 3B]. We found a total of 14 genes that were differentially expressed (adj < 0.05) commonly between both the RR cell lines, including seven genes (TNS4, ZDHHC8P1, APLNR, AQP3, SPP1, ID1, ID2) upregulated, and seven genes (PTX3, ITGB2, EPS8L1, ALDH1L2, KCNT2, ARHGAP9, IFI16) that were downregulated.

The full list of genes differentially expressed in both cell lines, function, mutational rate in PDAC, and prior studies examining radiation sensitivity is displayed in Table 1. Most of these genes have some implications for radiation sensitivity, and the gene with multiple studies relating to RR was the ID1 gene. We next analyzed the publicly available cancer cell encyclopedia (CCLE) database, which has IC_{50} treatment response

Table 1. Significantly upregulated genes in both radiation resistant pancreatic cell lines

Significantly upregulated genes				
Gene ID	Gene name	Cellular function*	Mutational frequency in pancreatic cancer^	Association with radiation
APLNR	Apelin receptor	A member of the G protein-coupled receptor gene family. The encoded protein is related to the angiotensin receptor	5.5%	None
AQP3	Aquaporin 3	The water channel protein aquaporin 3. Aquaporin 3 is localized at the basal lateral membranes of collecting duct cells in the kidney	4.6%	AQP3 expression is downregulated by UVA irradiation[15]
ID1	Inhibitor Of DNA binding 1	A helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors. The encoded protein has no DNA binding activity and therefore can inhibit the DNA binding and transcriptional activation ability of basic HLH proteins with which it interacts	3.7%	1) In Glioblastoma, PGE2-mediated induction of ID1 is required for optimal tumor cell self-renewal and radiation resistance[16]. 2) ID1 overexpression in GBM cells increased radioresistance[17]. 3) Id1 and Id3 co-expression seems associated with a poor clinical outcome in patients with locally advanced NSCLC treated with definitive chemoradiotherapy[18]. 4) In GBM, a significant correlation ($P < 0.001$) was found between radiotherapy efficacy and ID1 expression levels with respect to overall survival and knockdown of ID1 increased radiosensitivity *in vitro*[19]
ID2	Inhibitor Of DNA binding 2	A helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors. The encoded protein has no DNA binding activity and therefore can inhibit the DNA binding and transcriptional activation ability of basic HLH proteins with which it interacts	0.9%	ID2 is induced in response to γ-irradiation[20]
SPP1	Secreted phosphoprotein 1	Involved in the attachment of osteoclasts to the mineralized bone matrix	1.8%	SPP1 regulates radiotherapy sensitivity of gastric adenocarcinoma via the Wnt/Beta-Catenin pathway[21]
TNS4	Tensin 4	Involved in protein localization. Located in focal adhesion	1.4%	None
ZDHHC8P1	ZDHHC8 pseudogene 1	Pseudogene	NA	None

*https://www.genecards.org/; ^https://www.cbioportal.org/.

data for the DNA damaging agent 5-Fluorouracil (5-FU) from 600 cancer cell lines including 27 PDAC cell lines. We compared the mRNA expression in the CCLE from each of the significant genes found in our RNA-seq experiment [Supplementary Figures 1 and 2]. We plotted mRNA expression against the IC_{50} value for 5-FU. We found ID1 expression had the highest correlation and strongest *P*-values with higher treatment resistance to 5-FU using Spearman and Pearson correlation tests. These results strengthen our findings that ID1 is a marker for resistance to DNA damaging agents in PDAC. Therefore, we further characterized the ID1 gene as a potential mediator of RR in PDAC patients.

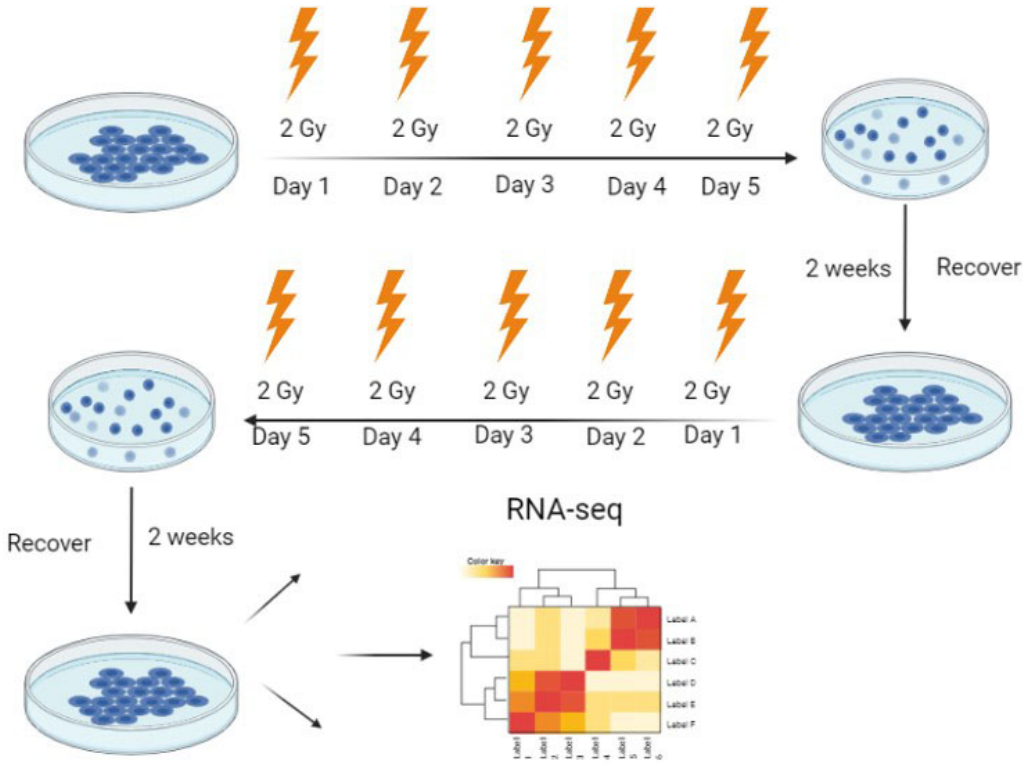

Figure 1. Generation of radioresistant (RR) PDAC cell lines. Schematic of the generation of RR sublines. Cell lines were exposed to 5 fractions of 2 Gy irradiation over one week and then allowed to recover for two weeks, followed by a second course of 5 fractions of 2 Gy over one week and an additional 2-week recovery period. Subsequently, RR cells were collected for RNA-seq analysis, western blotting, and sensitivity to radiation using the clonogenic assay to compare the parental and RR sublines and created with BioRender.com.

ID1 expression is increased following RT resistance

Following the generation of RR cell lines, we collected the protein lysates of the control and RR cell lines to test the expression of the ID1 protein. Western blotting revealed that ID1 protein expression was increased in the PANC-1 and MIA PACA-2 RR cells compared with parental PANC-1 and MIA PACA-2 cells [Figure 4A and B]. These results confirm the RNA-seq data showing increased ID1 levels in both RR cell lines compared to the parentals. We next sought to address the mechanism of ID1 and radiation resistance. We knocked down the expression of the ID1 protein through siRNA transfection of both the parent and RR PANC-1 cells. After 48 h of siRNA treatment, good knockdown was shown in both cell lines [Figure 4C]. We next performed clonogenic experiments comparing radiation sensitivity in the parent and RR cells following siControl or siID1 pre-treatment. We again show that RR cells display radioresistance compared to the parental cells. We found that siID1 transfection of the RR cells reverted the sensitivity to radiation of the RR cells compared to the siControl parental cells. There was no impact on the radiation sensitivity of the parental cells following siID1 transfection [Figure 4D]. These results suggest that ID1 is activated in the radiation resistance population, and targeting ID1 could be a strategy to increase sensitivity to radiation.

ID1 is a potential biomarker in PDAC patients

To evaluate the expression profile of ID1 in PDAC, we analyzed the available tissue sample data from both IHC and RNA-seq data from PDAC patients in the TCGA project and normal samples in the GTEx project using the human protein atlas website. As shown in Figure 5A, ID1 high or medium protein expression was

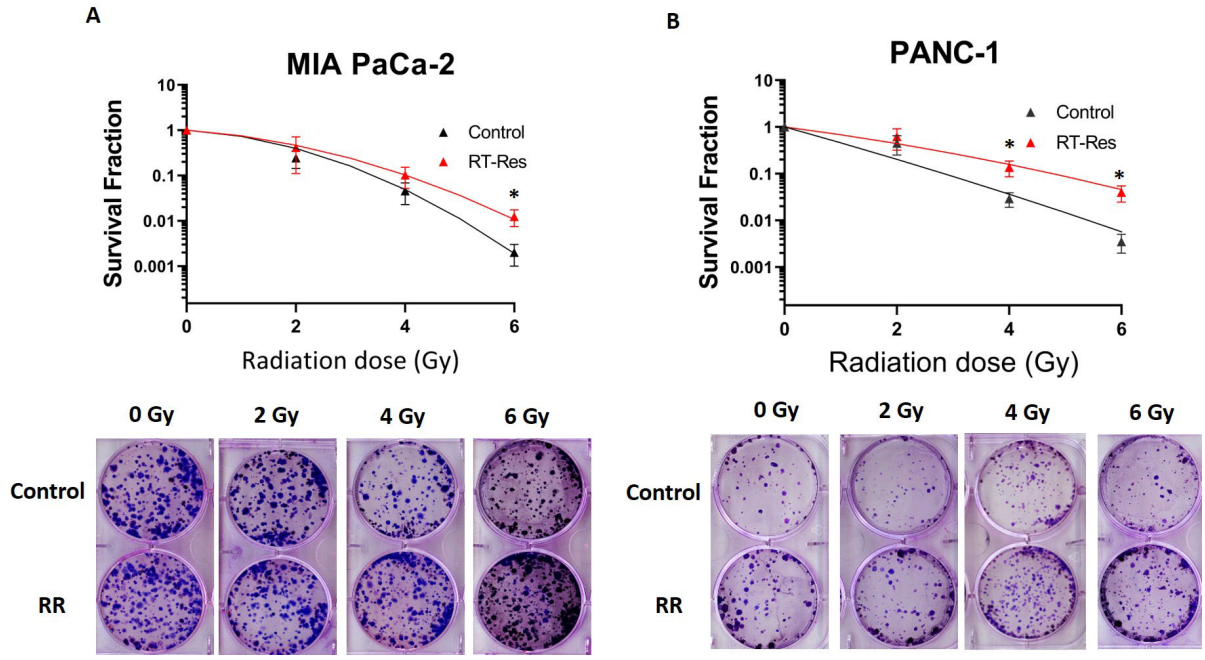

Figure 2. RR PDAC sublines display increased resistance to radiation. Colony formation assay of (A) MIA PACA-2 RR *vs.* MIA PACA-2 and (B) Panc-1 RR *vs.* Panc-1. Representative images of colony formation following increasing doses of IR are displayed below the curves. *$P < 0.05$; RR: radioresistant.

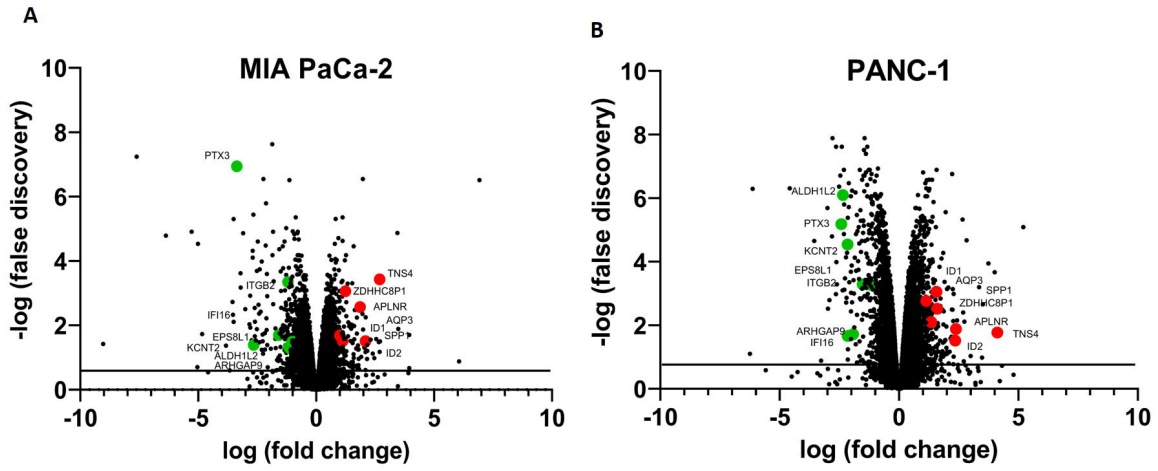

Figure 3. RNA-seq analysis reveals gene expression differences between radioresistant cells and parental cells. Volcano plot representation of differential expression analysis of genes in the parental cells versus RR cells for the MIA PaCa-2 (A) and PANC-1 (B) cells. Red and green points mark the genes with significantly increased or decreased expression in both MIA PaCa-2 and PANC-1 cell lines (FDR < 0.05). The x-axis shows log fold changes in expression, and the y-axis is the negative log of the false discovery value.

found in 75% of pancreatic cancer patients, and PDAC was the second-highest ID1 expressing cancer behind only thyroid cancer. In the normal tissue samples from the GTEx RNA-seq dataset, the normal pancreas had relatively low expression compared to other tissues. The pancreas was the 4th lowest normal tissue expression [Figure 5A]. The direct comparison of ID1 mRNA expression in PDAC tissue *vs.* normal pancreas tissue showed significantly higher ID1 expression in the tumor samples compared to normal tissues ($P < 0.05$) [Figure 5B].

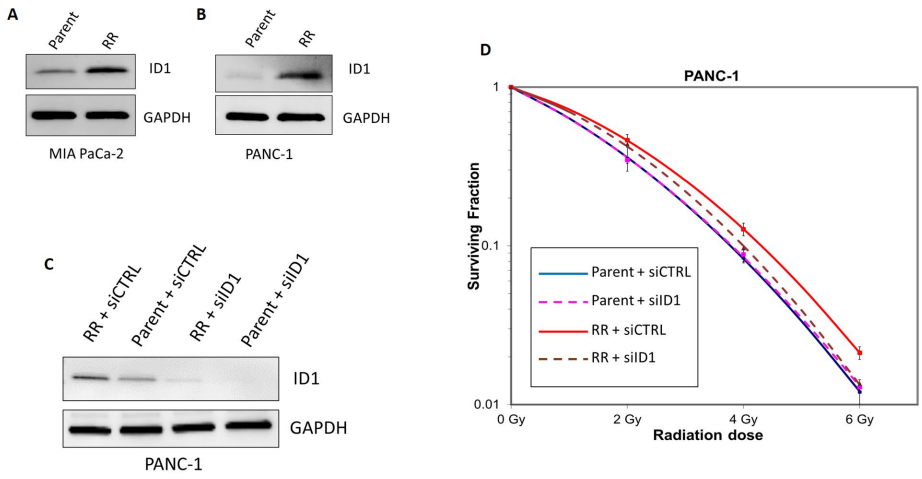

Figure 4. ID1 expression is increased in radioresistant cells. Western blotting confirmed increased protein expression of the ID1 gene in RR cells compared to the parental controls in both (A) MIA PaCa-2 and (B) PANC-1 PDAC cell lines. (C) Western blotting of ID1 and GAPDH 48 h following siRNA transfection of either control or ID1 in parental or RR PANC-1 cells. (D) Clonogenic curves following siControl or siID1 transfection of parent and RR PANC-1 cells. RR: Radioresistant; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

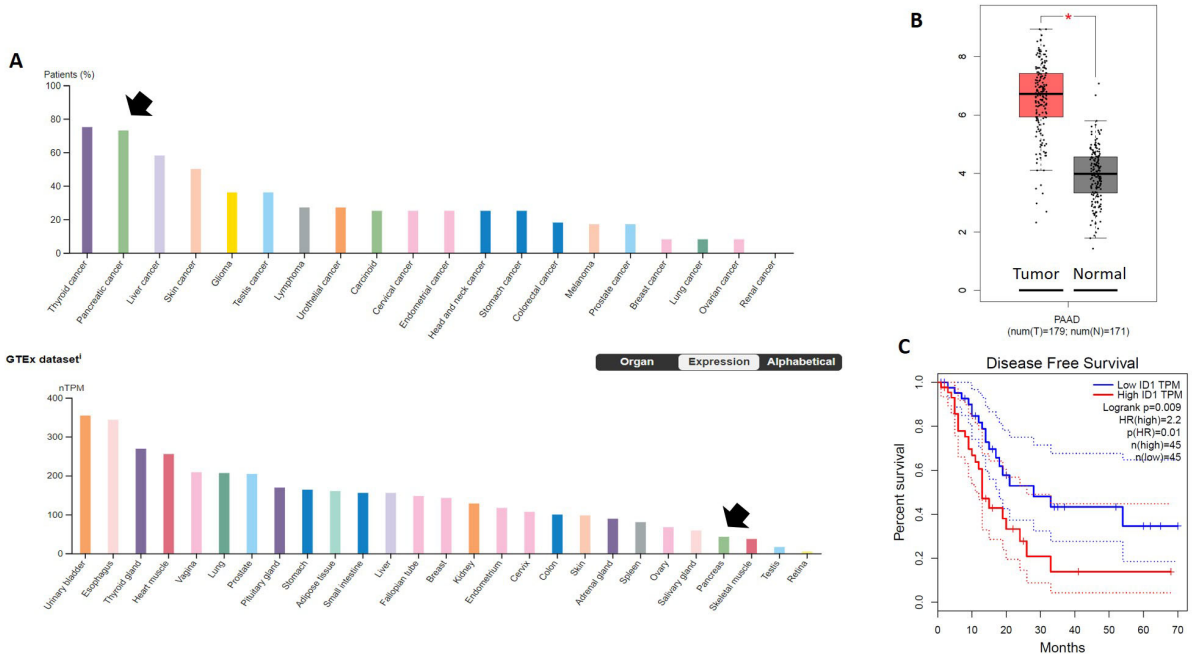

Figure 5. ID1 expression is high in PDAC tissue compared to normal samples and correlates with worse disease survival. (A) The percent of tumor samples with an either high or medium expression of ID1 on immunohistochemistry (IHC) was rank-ordered. The mRNA expression of ID1 in normal tissue was rank-ordered in normal tissue samples from the GTEx RNA-seq dataset. Arrow points to PDAC and pancreas tissue. (B) Direct comparison of mRNA expression data from PDAC tumors in the TCGA and normal pancreatic tissue from GETx. *$P < 0.05$. (C) PDAC patients in the TCGA dataset were grouped based on mRNA expression of ID1 split on the median value. High ID1 tumor expression predicted worse disease-free survival, $P = 0.009$ HR = 2.2.

We next investigated the association between ID1 expression and disease-free survival (DFS) from the TCGA database. Survival analysis using Kaplan-Meier and log-rank test divided at the median value revealed lower DFS in PDAC patients with high ID1 expression compared to patients with low ID1

expression [hazard ratio (HR) = 2.2, P = 0.009] [Figure 5C].

The p53 gene is a known regulator of cellular response to radiation therapy[22]. Patients with mutated p53 in multiple cancer types, including PDAC, have worse RT response rates than p53 wildtype patients[23]. From our analysis of the TCGA PDAC cohort, p53 mutant patients had lower DFS than p53 wildtype patients (log rank P = 2.9e-3) [Figure 6A]. We next sought to determine if there was a correlation between p53 mutation and ID1 expression. In the TCGA tumor dataset, we found that p53 mutant PDAC patients had significantly higher ID1 mRNA expression than p53 wildtype patients (P = 0.03) [Figure 6B].

Together, our data showed that expression of ID1is upregulated in PDAC and is associated with poor survival and p53 mutation, supporting ID1 as a potential mediator in PDAC RR.

DISCUSSION

Although the development of metastatic disease dominates the natural history of PDAC, local tumor progression contributes significantly to morbidity and mortality. Conventional RT (cRT) with small 1.8-2 Gy/fraction doses has shown low long-term tumor control rates or survival benefits. Over the past decade, dose-escalation strategies (otherwise termed ablative RT, or aRT (3-4 Gy/fraction)), have shown promising results[24-27]. The aRT technique requires a highly specialized team of Radiation Oncologists and Physicists in addition to state-of-the-art technologies such as daily adaptive MR linear accelerators, only available in a handful of centers in the U.S.A. Outside of large tertiary cancer centers, delivery of aRT while safely avoiding normal tissue dosing is possible for a minority of PDAC patients where tumors are far enough away from the luminal gastrointestinal (GI) tract. Therefore, novel strategies to improve radiosensitivity to standard lower doses of RT are critical. To enhance radiosensitivity, genetic drivers of RR are crucial to be discovered to develop novel targeted therapeutics to combine with RT.

Two previous studies have studied gene regulation of radiation resistance using engineered radioresistance PDAC cell lines. Souchek *et al.*[28] and Ogawa *et al.*[29] used the cDNA microarray method to identify gene changes following radioresistance. The Soucheck study found the cholesterol pathway to be significantly upregulated in radioresistance cells, including genes: FDPS, ACAT2, AG2, CLDN7, DHCR7, ELFN2, FASN, SC4MOL, SIX6, SLC12A2, and SQLE. The Ogawa study found upregulated pathways related to growth factors, cell cycle checkpoint, and angiogenesis, including genes: AREG, MAPKAPK2, RGN, ANG-2. Our study generated RR PDAC cells and utilized the RNA-seq to further increase the current understanding of gene expression changes in radioresistant PDAC cell populations. The novelty of our study is that we used a clinically relevant dose fractionation of 2 Gy/day to generate our RR cell lines compared to a single 10 Gy dose in the Ogawa study. The Soucheck study also used this clinically relevant dose fractionation, but in contrast to our study, it only utilized one cell line. In this study, two different PDAC cell lines were generated, and genes that were upregulated in both were analyzed further, increasing the robustness of the current study.

Other studies have examined intrinsic radiation sensitivities in various PDAC cell lines to study potential therapeutic vulnerabilities. Wiechmann *et al.* screened 38 PDAC cell lines and pulled out two radiation sensitive and two resistant cell lines to study the phosphoproteomes in response to 8 Gy of radiation[30]. They found increased actin dynamics and FAK activity in the resistant cell lines and FAK inhibition radiosensitized radioresistant cell lines more than the radiosensitive cell lines. Schröter *et al.* examined the role of high dose radiation and changes in cell surface expression of immunomodulatory molecules[31]. Interestingly, they found that only high radiation doses of > 5 Gy increased surface expression of PD-L1 and CD73. These results have implications for the potential use of immunotherapy combinations with radiation

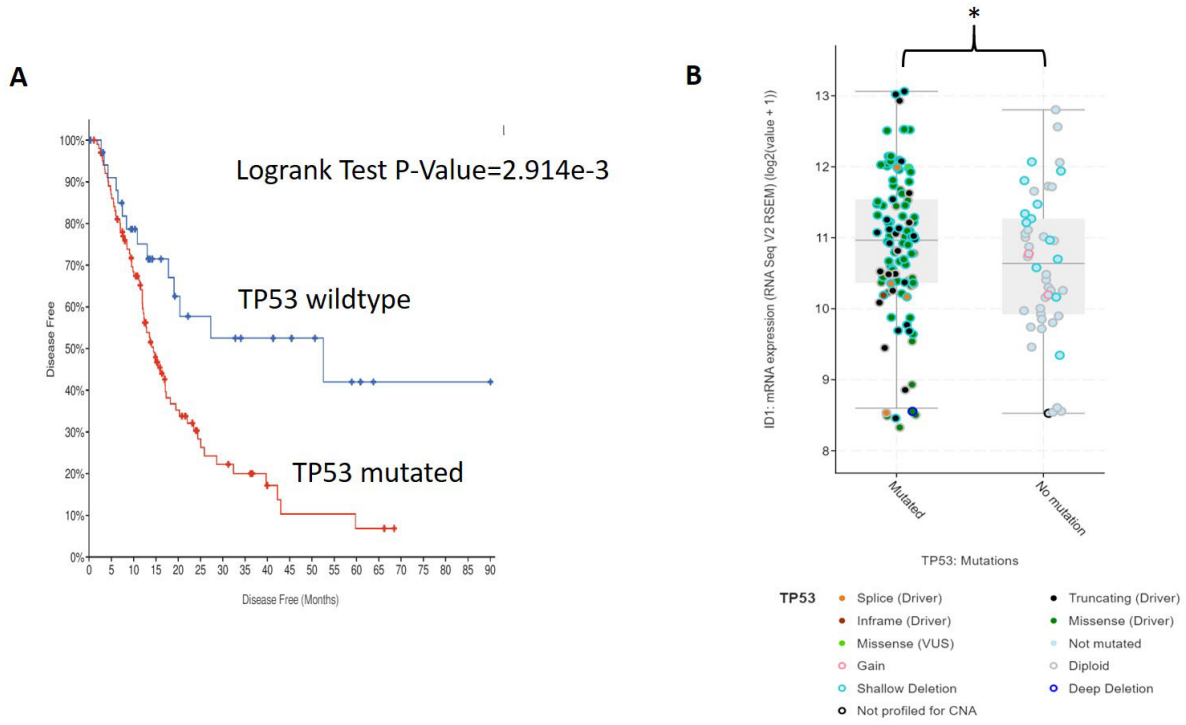

Figure 6. TP53 mutation is a poor prognostic factor and correlates with higher ID1 expression. (A) PDAC patients in the TCGA dataset with tumor p53 mutation (63%) were compared to p53 wildtype for disease free-survival (DFS). Patients with p53 mutations had significantly worse DFS than p53 wildtype, $P = 0.003$. (B) ID1 mRNA expression was compared in p53 mutated tumors *vs.* p53 wildtype tumors from the TCGA PDAC dataset. ID1 expression was higher in the p53 mutated patients $*P < 0.05$.

such as stereotactic body radiotherapy (SBRT) which utilizes high doses per fraction of radiation.

In our study, we generated new RR PDAC cell lines and then further characterized the changes in gene expression using next-generation-based RNA-Seq. The new cell lines displayed higher survival levels after radiation *in vitro*, and we discovered 14 genes that had significant changes in expression in the RR cell lines compared to the parental. Of these genes, we explored one gene, ID1, that has multiple prior published reports in other cancer types related to RR.

ID1 is a helix-loop-helix (HLH) protein that can form heterodimers with members of the basic HLH family of transcription factors. The encoded protein has no DNA binding activity and, therefore, can inhibit the DNA binding and transcriptional activation ability of essential HLH proteins with which it interacts[32]. Maruyama *et al.* reported that PDAC cells expressed high levels of the ID genes and that ID1 expression was increased compared to normal controls. The authors concluded that increased ID1 expression might be associated with the enhanced proliferative potential of pancreatic cancer cells[33]. In our analysis of the TCGA database, we found that ID1 expression was highly expressed in pancreatic tumor samples compared to normal pancreas tissue [Figure 5].

There have been several prior published publications examining the role of ID1 in radiation resistance, reviewed in Table 1. Cook *et al.* showed that PGE2 signaling regulated radiation resistance in mouse glioblastoma (GBM) primary cultures in an ID1-dependent manner[16]. In GBM, ID1 overexpression increased radioresistance[17], and ID1 expression levels predicted poor overall survival and knockdown of ID1 increased radiosensitivity *in vitro*[18]. One of the other genes from our RNA-seq data shown to regulate

radiation sensitivity was SPP1 in gastric adenocarcinoma[21].

To explain the potential role of ID1 in associating with RR in PDAC cells, we found that patients with p53 mutation had significantly higher expression of ID1 mRNA than p53 wildtype patients. Qian and Chen reported that ID1 expression was downregulated following DNA damage in a p53-dependent manner. They found that ID1 overexpression promoted cell proliferation and inhibited DNA damage-induced cell senescence. They concluded that ID1 was a critical p53-dependent DNA damage response pathway[34].

Looking into the future, ID1 may serve as a therapeutic target to increase the radiosensitivity of PDAC tumors and improve the outcomes in PDAC. Further studies developing strategies to target ID1 are warranted.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study and performed data analysis and interpretation: Zuniga O, Byrum S, Wolfe AR

Performed data acquisition, as well as provided administrative, technical, and material support: Wolfe AR

Availability of data and materials

All models are available to academic centers under an institutional MTA.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Experiments were conducted using protocols and conditions approved by the Institutional Biosafety Committee at UAMS (PROTOCOL #: BP370).

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Aberrant Notch signaling in gliomas: a potential landscape of actionable converging targets for combination approach in therapies resistance

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Abstract

The current therapeutic protocols and prognosis of gliomas still depend on clinicopathologic and radiographic characteristics. For high-grade gliomas, the standard of care is resection followed by radiotherapy plus temozolomide chemotherapy. However, treatment resistance develops due to different mechanisms, among which is the dynamic interplay between the tumor and its microenvironment. Different signaling pathways cause the proliferation of so-called glioma stem cells, a minor cancer cell population with stem cell-like characteristics and aggressive phenotype. In the last decades, numerous studies have indicated that Notch is a crucial pathway that maintains the characteristics of resistant glioma stem cells. Data obtained from preclinical models indicate that downregulation of the Notch pathway could induce multifaceted drug sensitivity, acting on the expression of drug-transporter proteins, inducing epithelial–mesenchymal transition, and shaping the tumor microenvironment. This review provides a brief overview of the published data supporting the roles of Notch in drug resistance and demonstrates how potential novel strategies targeting Notch could become an efficacious action to improve the therapy of high-grade glioma to overcome drug resistance.

Keywords: Tumor microenvironment, notch inhibitors, stem cells, brain tumors, drug resistance

INTRODUCTION

The most frequent malignant primary brain tumor in adults is glioma. Low-grade glioma (LGG) includes WHO grades 1-2, while high-grade glioma (HGG) includes WHO grades 3-4. Glioblastoma (GBM), one of the most lethal and most prone to recurrence of malignant solid tumors, represents the majority of WHO grade 4. GBM accounts for more than 50% of all gliomas and primary malignant brain tumors[1]. Current molecular diagnostic techniques allow alternative classification based on molecular abnormalities and signaling pathways involved in glioma genesis. Isocitrate dehydrogenase (IDH) is a basic biomarker for subtyping and prognosis of gliomas. Tumors that are IDH-mutant have a better prognosis than their counterparts with similar histologic grade and IDH-wildtype phenotype[2]. Furthermore, methylation of the O6-methylguanine DNA methyltransferase (MGMT) promoter is related to a better response to chemotherapy with temozolomide (TMZ) and longer overall survival[3]. However, the combination of multiple tumorigenic events, such as highly deregulated tumor genome with hyper-activation of oncogenes, causes and sustains the pathogenesis of glioma and resistance to various therapies[4]. For instance, oncogenic activation of receptor tyrosine kinases (RTKs) is a crucial signaling mechanism for maintaining glioma oncogenicity. Among all the RTK alterations, epidermal growth factor receptor (EGFR) gene amplification is the most common variation (approximately 40%) in GBM, although EGFR inhibitors (e.g., gefinitib and erlotinib) have not caused clinical effects in patients with GBMs in clinical trials[5]. Increased expression of platelet-derived growth factor receptors (PDGFR) and PDGF has been evidenced in astrocytic cancers of different grades and is associated with poor prognosis[6]. Although GBM patients present sporadic RAS alterations, RAS and AKT mediate fundamental pathways in different models of GBM[7]. In addition, Sonic Hedgehog homolog (SHH), which mediates molecular signals to the embryonic cells required for normal development and Notch, plays a crucial role as an important factor influencing neural progenitor behavior and is dysregulated in glioblastoma stem cells (GSCs)[8]. In the next sections, we recapitulate the state of our knowledge regarding the functional role of the Notch signaling pathway, focusing on GBM disease progression and therapy resistance and suggesting novel treatments to overcome drug resistance for effective therapeutic protocols of brain malignancies.

NOTCH SIGNALING

The Notch receptor mediates a highly conserved signaling pathway that is crucial in central nervous system development and malignant transformation[9,10]. Four Notch receptors (Notch1 to Notch4) interact with different canonical activators such as delta/serrate/lag-2 (DSL) ligands Jagged1 and Jagged2 and delta-like (Dll) 1, Dll3, and Dll4 of the Notch pathway[11]. Notch1 and Notch2 are extensively expressed in numerous tissues during embryonic development and in adult mammals[12]. Vascular smooth muscle and pericytes express elevated Notch3, while Notch4 is much more abundant in the endothelium[13,14]. Notch receptors, located on the cell membrane[15], are characterized by N-terminal EGF repeats that compose the extracellular portion and by a juxtamembrane negative regulatory region (NRR). The intracellular region is formed by a transcriptional activation domain and a C-terminal degron domain rich in the amino acids proline, glutamate, serine, and threonine (PEST)[13,16]. Notch signaling is initiated by the contact of DSL ligands from sending cells and Notch receptors existing on proximal cells. After this occurrence, the Notch protein undergoes additional proteolytic cleavage determined by the γ-secretase enzyme complex, originating in the Notch intracellular domain (NICD) which translocates into the nucleus. NICD binds to DNA/chromatin-specific sequences, displaces a repression complex, interacts with the DNA binding protein recombination signal binding protein for immunoglobulin kappa j region (RBPJ), and recruits the co-activator mastermind-like1 (MAML1)[17]. Next, the ternary complex recruits the histone

acetyltransferases P300/CBP-associated factor (PCAF), a key regulator of transcription which is expressed in GBM cells[18]. Lastly, these protein complexes induce the expression of Notch-regulated genes, for example, hairy enhancer of split (Hes) and HES-related proteins (Hey), both important in lineage-commitment choices. In addition, cell cycle regulators such as p21/Waf1, CYCLIN D1 (CD1) and CD3, and an important regulator of stem cell biology (c-Myc), an EGFR-related gene found to be amplified in human breast cancer cell lines such as the human epidermal growth factor receptor 2 (HER2), as well as nuclear factor kappa B (NF-κB), insulin-like growth factor 1-receptor (IGF1-R), survivin, snail homolog 2 (SLUG), SOX2, and paired box (PAX) 5[19,20], all directly associated with tumorigenesis, are described as Notch target genes[20,21]. The Notch-dependent transcriptional activity concludes with NICD degradation. Cyclin-dependent kinase 8 (CDK8), which binds to and/or phosphorylates several transcription factors, causes the phosphorylation of a degron within the PEST domain of NICD and is targeted for proteasome-mediated degradation by E3 ubiquitin ligases SEL10 (FBW7)[15]. The Notch pathway may be regulated through post-translational modification[22], which changes the affinity of the Notch receptor for DSL. The stability of NICD and the extent of signaling are also influenced by phosphorylation of Notch proteins induced by glycogen synthase kinase 3β (GSK3β), largely expressed throughout the brain with an additional expression on endothelial cells in mice[23]. Beyond the established signaling stimulation, distinct proteins missing the DSL domain have been described[24], such as membrane-integral proteins delta/Notch-like epidermal growth factor-related receptor (DNER), a glycosylphosphatidylinositol (GPI)-linked membrane (e.g., NB3/Contactin6), or secreted proteins[25]. For instance, NICD physically cooperates with a major component of canonical WNT signaling, β-catenin[26], Smad tumor suppressor proteins[27], and the key regulators of cellular response to changes in oxygen concentration, such as hypoxia-inducible factor 1 alpha (HIF-1α)[28], thus obtaining direct crosstalk between Notch and the wingless/integrated (WNT), transforming growth factor-beta (TGF-β), and hypoxia-dependent signaling pathways.

NOTCH SIGNALING IN CANCER

Given the outstanding position of Notch in regulating cellular behavior, it is well recognized that Notch signaling is altered in a wide range of diseases, including human malignancies. Unbiased genome-scale sequencing data indicate mutations in Notch genes in various types of cancers. Interestingly, the positions, identities, and effects of these mutations represent varied roles for Notch in different types of cancers[29], such as breast cancer[30], prostate cancer[31], lung cancer[32], glioblastoma[33], and other malignancies[34]. Definitely, functional studies consider Notch signaling one of the hallmarks of cancer, although several data suggest both oncogenic and tumor suppressive functions, depending on the cancer type.

Three separate patterns of Notch gene mutations have been highlighted in several malignant tumors: (1) strong gain-of-function mutations that disrupt the NRR, with or without PEST degron domain deletions, as evidenced in T cell acute lymphoblastic leukaemia (T-ALL) and triple-negative breast cancer (TNBC); (2) PEST degron domain deletions only, as shown in B cell tumors; and (3) disruptive nonsense, frameshift, or point substitutions in the N-terminal portions of Notch receptors, which probably lead to loss of Notch function, as seen in squamous cell carcinoma, small cell lung carcinoma, bladder carcinoma, and certain low-grade gliomas[16]. A recent study focused on the frequent RBPJ copy number loss and diminished RBPJ protein expression in a significant minority of several types of carcinoma, mainly breast carcinomas[35]. Nevertheless, the connection between changed RBPJ gene dosage and tumorigenesis is multifaceted and may diverge depending on the tumor type.

The diverse mutational patterns of Notch receptor genes in specific cancers suggest that Notch can function as oncogenic signaling as well as a tumor suppressor in other situations[13]. The initial demonstration of an onco-suppressor action of Notch comes from results obtained in keratinocytes from mice and humans[36].

More recent data from whole-genome sequencing analysis describe Notch-inactivating mutations in patients with head and neck squamous cell carcinoma[37]. Thus, Notch signaling mediates both oncogenic and onco-suppressive action in similar tissue, as demonstrated in the hematopoietic system. Such a dual role is related to the action exerted by Notch in the modulation of cell fate decisions in immune cell expansion.

In addition, experimental data establish that the Notch signal has a fundamental part in cancer patient survival. For example, high expression of Jagged1 or Notch1 correlates with poorer overall survival when compared with low levels of both. Jagged1 was also found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues[38,39]. High levels of Jagged1 and Notch1 have been detected in breast cancers and were related to poor prognosis[38]. Shi *et al.* similarly reported that the Notch expression profile in papillary bladder transitional cell carcinoma appears dissimilar to the matched invasive phenotype. Consequently, Notch1 and Jagged1 may be used as additional markers for the prognosis of papillary bladder transitional cell carcinoma patients[40]. Cervical carcinomas patients positive for nuclear Notch3 expression showed shorter overall survival when compared with Notch3-negative patients; thus, Notch3 is a possible prognostic marker in cervical carcinomas[41,42]. Taken together, these data fuel additional scientific interest regarding the investigation of the Notch family as diagnostic and prognostic markers in human cancer.

NOTCH SIGNALING IS ABERRANTLY ACTIVATED IN BRAIN TUMORS

In recent years, large-scale genomic sequencing on brain tumors provided a unique understanding of the genomic aberrations and cellular signaling mechanisms that control brain tumor initiation and progression[43]. Among the different pathways, aberrant Notch signaling was found in brain tumors, even though its members are infrequently altered[43]. Elevated Dll1, Notch1, Notch3, Notch4, and HEY1 levels were associated with advanced glioma grade and poor prognosis[44,45], strongly suggesting that Notch signaling influences an undifferentiated and aggressive brain cancer phenotype. mRNA and protein levels of Notch1, Notch4, Dll1, Dll4, Jagged1, HEY1, HEY2, and HES1 are increased in brain tumors and associated with upregulation of vascular endothelial growth factor (VEGF) and phospho-AKT, together with a reduction of the tumor suppressor phosphatase and tensin homolog (PTEN)[33,46]. However, data suggest a controversial role of Notch1 in glioma genesis[47]. Specifically, Notch1 levels are increased in patients with a survival of > 1 year compared to < 1 year[48]. Thus, outstanding questions remain to be addressed. A very recent study suggested the Notch canonical ligand, Dll3, as a key therapeutic target and/or prognostic marker in LGG. The authors demonstrated that Dll3 promoter methylation and decreased mRNA expression influenced the patient's prognosis in LGG but not in GBM. This is a crucial discovery that identified subsets of LGG with different immune microenvironments which depend on Dll3 mRNA expression[49].

Notch signaling in glioblastoma drives GSCs phenotype

GBM harbors various cellular categories, several with amplified aggressiveness and stemness of GSCs, which are responsible for tumor relapse and therapy resistance[50,51]. GSCs are regulated by genetic, epigenetic, and metabolic factors, in addition to extrinsic factors, the immune system, and the tumor microenvironment (TME)[52]. Indeed, vascular cells, microglia, peripheral immune cells, and neural precursor cells that populate the TME control the course of the disease[53]. Specifically, the multiple molecular interactions of bulk glioma cells and GSCs with the TME exert a pathological impact influencing tumor progression and response to different treatments[53]. These observations suggest targeted therapeutic approaches that are effective against heterogeneous GBM cell populations[54].

The potent tumorigenic activity of GSCs has been associated with the expression of Nestin, glial fibrillary acidic protein (GFAP), β-III tubulin, and CD133 stem cell markers[55]. Among the different aberrant signaling pathways, the Notch pathway, co-opted in GBM, stimulates astrocytes to assume a stem-like state through different mechanisms (as summarized in Figure 1), which can be accompanied by increased proliferation[56]. The dependence of GSCs on Notch signaling is further supported by experiments demonstrating depletion of GSCs by treatment with γ-secretase inhibitors[57]. Interestingly, cell fate determinant Numb4 controls the expression of stem cell markers in GSCs, which function as regulators of Notch signaling. Overexpression of Numb4 decreased EGFR expression due to Numb-mediated endocytosis[58].

Active Notch signaling is essential for maintaining the tumorigenic potential of GSCs and drug resistance under hypoxic conditions. Accordingly, stimulation of the Notch pathway by HIF-1α in GSCs is crucial for hypoxia-mediated effects. Indeed, reduction of HIF-1α or inhibition of Notch signaling partially prevents the hypoxia-mediated maintenance of GSC[59].

Notch effects are also evident in terms of metabolic reprogramming, which is mainly influenced by mitochondrial respiration. Reverse electron transfer (RET), which influences ROS levels under a variety of metabolic conditions, many of which are associated with pathology, is particularly active in GCSs. Notch modulates RET through the interaction with specific respiratory chain complex I (RC-I) proteins. The authors demonstrated that genetic and pharmacological interference of Notch and RET inhibited GSC proliferation in mouse models. These results demonstrate the crucial role of Notch as a regulator of metabolic reprogramming of brain tumors, suggesting novel therapeutic purposes[60] targeting the metabolic regulation of GBM cells. Besides, further data support the multifaceted role of Notch. On the one hand, Notch2 expression levels are associated with Nestin and SOX2 stemness markers, together with vimentin and GFAP, as well as anti-apoptotic proteins, but they are also inversely associated with pro-apoptotic proteins in GBM tissue[33]. On the other hand, several groups have reported dissimilar expressions of Notch1, Notch2, MAML1, and pPCAF300 in human glioma, with controversial results regarding tumor progression and prognosis[47].

Notch Signaling drives drug resistance and disease progression dependent on GSCs

In GBM, innate and acquired mechanisms of treatment resistance could depend on the blood - brain barrier, tumoral heterogeneity, and TME. In addition, GSCs are actually retained to support GBM progression and resistance to different drugs[61]. Different mechanisms have been described, and, more recently, a live-imaging study of GSCs acquired from the infiltrative area and putative relapse-driving area showed that GSCs are interconnected and able to transfer mitochondria via thin membranous open-ended channels, connecting distant cells. The authors concluded that this novel mechanism might have a potentially relevant role in therapy resistance; however, the increased stem cell population, DNA repair activity, expression of multidrug transporters, and redundant signaling pathways such as Notch[62] are better established and investigated mechanisms of resistance (as summarized in Figure 2). Data from different experimental models suggest that anticancer drugs such as TMZ[63], bevacizumab[64], and oxaliplatin[65,66] frequently induce Notch, which in turn controls the factors implicated in drug efflux, metabolic reprogramming, regulation of GSCs and TME, etc. that lead to acquired resistance. Loss of serine/threonine-protein kinase polo-like kinase 2 (PLK2), a key regulator of centriole duplication, was found to be strongly related to acquired resistance to TMZ via stimulation of the Notch pathway in GBM. Particularly, the authors suggested that loss of PLK2 induced the Notch pathway, mediated by the transcriptional inhibition of HES1[63].

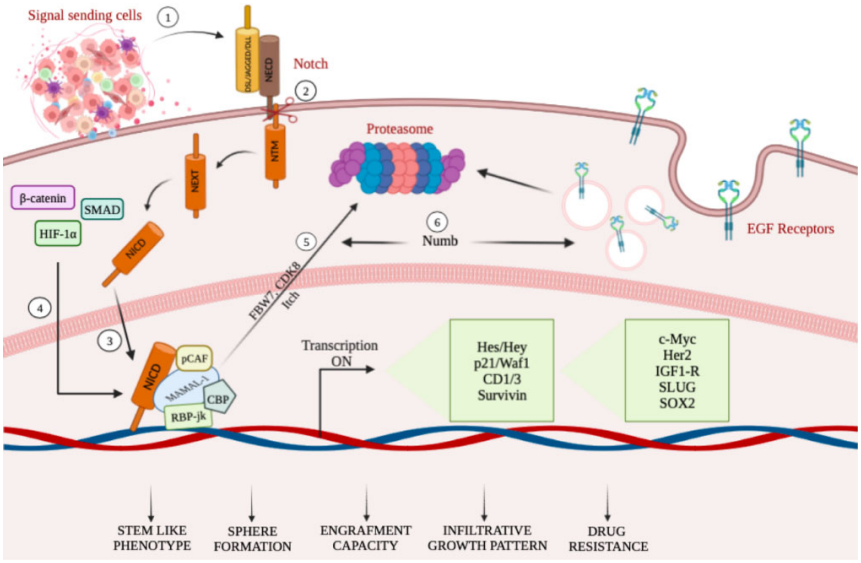

Figure 1. A schematic summary illustrating mechanisms targeting Notch in GCSs (see the text for details). (1–3) TME cell populations, secreting a number of factors including Notch ligands, stimulate the transcriptional activity of Notch receptors, thus influencing tumor biology. (4) NICD physically cooperates with β-catenin, Smad proteins, and HIF-1α, thus obtaining the crosstalk among Notch, Wnt, TGF-β, and hypoxia-dependent signaling pathways. (5) Notch-mediated transcriptional activation achieves the NICD degradation through phosphorylation of NICD, mediated by CDK8 and targeted for proteasome-mediated degradation. (6) Decrease of EGFR expression due to Numb-mediated endocytosis.

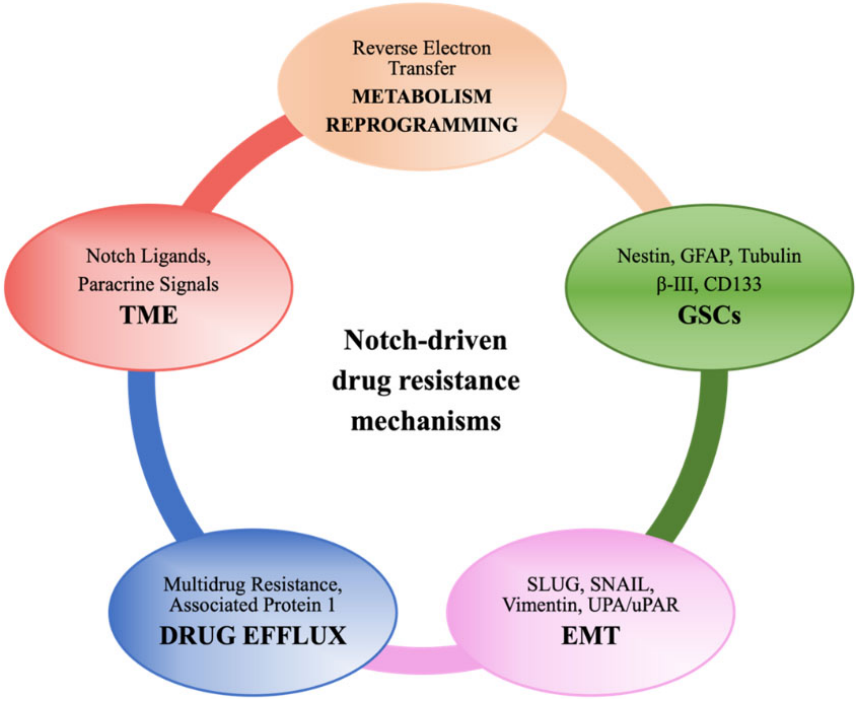

Figure 2. A schematic illustration showing Notch-driven drug resistance mechanisms (see the text for details).

Several fundamental mechanisms have been described; for example, Notch1 regulates the expression of multidrug resistance-assosicated protein 1 (MRP1), which plays an intrinsic role in the chemoresistance of GBM tumor cells[67]; thus, Notch is an encouraging target for the improvement of the current glioma

therapeutic portfolio. Blocking Notch signaling or RBPJ reduced clonogenic potential in tumor-sphere assays and engraftment capacity in GBM xenograft models[68]. Accordingly, NICD overexpression could induce cell survival due to radioresistance and side population phenotype in glioma cells[69]. Moreover, the extracellular matrix glycoprotein Tenascin-C and Jagged1 strengthen the expression of each other, promoting brain tumor-initiating cell (BTIC) growth. These cells from human GBM patients exhibit increased resistance to radiation and chemotherapy[70].

GSC population enrichment and its increasing resistance to treatment are favored by the brain TME, secreting a number of factors including Notch ligands which influence tumor biology. For instance, endothelial cells provide ligands, bind the Notch receptors, and are expressed by GSCs. Paracrine signals from mesenchymal stem cells (MSCs) induce chemosensitization to TMZ in heterogeneous GSCs that lack EGFR amplification through the downregulation of Notch1 and SOX2 and the upregulation of vimentin[71].

The poor prognosis of GBM patients and the high levels of radio- and chemoresistance are strictly associated with the highly infiltrative capacities of these tumors. Primary GBM comprises both proneural and mesenchymal GSCs, but the treatment causes proneural to mesenchymal transition, determining the prevalence of mesenchymal GSCs in resistant tumors[72]. The mechanisms are analogous to those underlying the epithelial–mesenchymal transition (EMT). SLUG expression, which is linked to the mesenchymal profile, is regulated by the Notch1 pathway and has been implicated in radioresistance[73]. An elegant study demonstrated that GBM-derived neurosphere cultures with elevated Notch levels were prone to form intracranial tumors with major infiltrative characteristics. In this scenario, elevated Notch1 expression confers a more invasive phenotype to neurosphere cultures once matched to the low Notch1 experimental condition[74]. Downregulation of urokinase-type plasminogen activator/urokinase-type plasminogen activator receptor (uPA/uPAR), a multifunctional system playing a critical role in GBM invasion, inhibited the cleavage of the Notch receptor, thus inhibiting Notch signaling-induced AKT, NF-κB, and extracellular signal-regulated kinases (ERK) pathways[75].

TARGETING NOTCH SIGNALING IN THE EMERGENCE OF RESISTANCE TO CURRENT GLIOBLASTOMA THERAPIES

The existence of the GSC population within the tumor is an important limiting factor for achieving therapeutic success for GBM patients. These GSCs possess increased resistance to chemo- and radiotherapy compared to bulk cancer cells, although they appear to be sensitive to treatment targeting Notch[57,76]. However, additional difficulties limit the therapeutic response of GBM patients, such as insufficient drug delivery across the blood–brain barrier, abundant intra- and intertumoral heterogeneity, and an immunosuppressive microenvironment. The therapeutic resistance in GBM is particularly complicated by the interplay between GSCs and the TME, which is crucially orchestrated by the Notch pathway[77], suggesting that the novel therapeutic approach targeting Notch could overcome drug resistance. In the subsequent sections, we review the state of our knowledge regarding the functional role of Notch signaling pathway in drug resistance and several approaches that are potentially valuable to overcome drug resistance for the efficacious treatment of gliomas.

Direct and alternative targeting of Notch signaling

Starting from the molecular structure of Notch receptors, Notch ligands, and Notch activators, several studies have aimed to target the Notch pathway pharmacologically. The majority of identified compounds revealed anticancer activity in several preclinical studies[78,79]. Moreover, the combined effects of several inhibitors with components of the current therapeutic portfolio are still under investigation in different clinical trials. Since the stimulation of Notch depends mainly on γ-secretase complex activity[80], γ-secretase

inhibitors, inhibiting NICD from Notch receptors, are the most promising in different solid cancers including GBM[81]. The inhibition of the second cleavage of the Notch receptor is possible through α-secretase inhibitors, which block members of the disintegrin and metalloproteinase (ADAM) family[82]. Additionally, novel promising therapeutic tools that directly or indirectly target Notch signaling have been developed to overcome therapy resistance.

Gamma secretase inhibitors

Several forms of γ-secretase inhibitors have been tested for anti-tumor effects, and DAPT is the first γ-secretase inhibitor tested in brain tumor experimental models[83,84]. DAPT stimulus in medulloblastoma xenografts induced apoptosis in cancer cells[85]. The positive effects of DAPT fortified the progress of γ-secretase inhibitors such as RO4929097, MRK-003, and L-685,458 (here indicated as GSIs)[86].

GSIs have been utilized in clinical and experimental studies in many tumors. Most of these studies have assumed that GSIs, except for potency, are biologic equivalents. However, the biology of GSIs is more complex, and the general assumption of pharmacological and functional equivalency of GSIs needs to be investigated further. Different studies designate several GSIs as the most clinically relevant anti-Notch treatments[87], even though the overall success in clinical trials shows limitations due to off-target complications[88]. These drugs have been analyzed on several subcutaneous brain tumor models, indicating the inhibition of neurosphere tumor growth by GSI-18. In established glial tumor models, Notch signaling downregulation using a single compound had partial effects, but it improved the efficacy of DNA-interfering agents. Accordingly, DAPT has been shown to improve the efficiency of TMZ[89]. Furthermore, DAPT has been shown to improve the efficiency of TMZ *in vivo*, as a single agent and in combination. Further studies in glial tumor models evaluated the GSI RO4929097 activity *in vitro*. Cotreatment with RO4929097 and chemotherapeutic agent improved TMZ properties and potentiated the cytotoxicity of etoposide and cisplatinum in glial tumor models. RO4929097 enhanced irradiation effects in GBM xenografts and decreased tumor growth in advanced-stage GBM xenografts. The authors concluded that the described preclinical data are necessary for the clinical development of Notch inhibitors in glial tumors[90]. Combined therapy with GSI RO4929097, radiation, and TMZ increased the survival of orthotopic GBM mouse models. The triple combination is more active than radio- and chemotherapy or GSI alone[91]. Similarly, DAPT intensifies the effects of radiation and decreases GSC growth and the number of endothelial cells disrupting the perivascular niche[92]. DAPT, tested in combination with an EGFR inhibitor, reduced endothelial cell sprouting and downregulated VEGF secretion. Therefore, the concomitant targeting of Notch and EGFR enhances the inhibitory effects on GBM angiogenesis and cell viability, suggesting a supplementary valuation of this targeting approach in clinical settings[93]. Similar to DAPT, other compounds, such as benzyloxicarbonyl-leu-leu-Nle-CHO (LLNle), induce GSC death and proteolytic stress and reduce NICD amount[94].

Furthermore, *in vivo* studies have shown that in the absence of supplementary therapies, less invasive tumors are generated from neurospheres isolated by xenografts previously treated with MRK003[95]. Similarly, a tripeptide with γ-secretase inhibitor activity may target CD133+ cells at a low concentration to serve as a radiosensitizer for GBM patients.

Some clinical trials have been directed to estimate the tumor-suppressive effects of a limited number of Notch inhibitors in recurrent invasive gliomas[88]. However, the lack of activity by RO4929097 and a marked reduction of steady-state drug levels have been described[96]. The cause for the absence of activity needs to be clarified. A possible mechanism could be the auto-induction of RO4929097 metabolism. A phase 0/I study evidenced the effect of chemo-radiotherapy in combination with RO4929097 in newly diagnosed GBMs. It

found the reduction of cells expressing CD133, a putative GSC marker. The authors concluded that, although evidence of target modulation was observed, recurrence occurred, associated with alterations in angiogenesis signaling pathways and variable blood–brain barrier penetration[79]. In a phase I/II trial, the combination of RO4929097 with bevacizumab targeting VEGF in patients with progressive or recurrent malignant glioma was tested. The drug combination was well-tolerated, and GBM patients indicated complete or partial remission after radiography[97].

α-secretase inhibitors

Notch is first cleaved by α-secretase outside the plasma membrane, via ADAM-10 and -17. A potent α-secretase inhibitor tested in GBM decreased cell proliferation and tumor size and prolonged survival *in vivo*. Furthermore, combination with DAPT reduced HES1 and HEY1 levels as well as leukemia inhibitor factor (LIF) and chitinase 3-like 1 (YsKL-40) levels, two new key players in GBM pathogenesis[82].

Antibodies

In addition to the secretase inhibitors, other molecules have been used to block Notch signaling in GBM, in order to overcome the drug resistance which drives disease progression. Blocking antibodies targeting specific isoforms of Notch receptors and engaged in contrast to the NRR, as well as blocking the receptor folding permitting ADAM cleavage, has been employed in preclinical and *in vitro* studies[98]. The specific antibodies, such as the anti-Notch1, proposed for GBM patients show the advantage of fewer side effects than GSIs. Another group of antibodies blocks Notch receptor - ligand interactions by hampering the EGF repeats essential for binding[99]. The first-in-human tested humanized anti-Notch1 blocking antibody treatment in a collection of GSC models with elevated Notch1 expression counteracted the transcription of Notch pathway target genes and caused significant impairment of cellular invasion under chemotherapy. However, the authors concluded that the observed phenotype could be due in part to Notch1 blockage as well as the activation of off-target signals[100]. Further pharmacological models were directed to Notch ligands such as for anti-Dll4 treatment. A rise in vessel density and reduced tumor dimension were observed after the administration of recombinant Dll4-Fc or anti-Dll4 polyclonal antibody in a bevacizumab-resistant human fibrosarcoma tumor model[101]. Similar findings were obtained in the human GBM cell line U87, subcutaneously implanted in nude mice. The authors showed that the overexpression of dominant negative soluble Dll4ECD-Fc enhanced the number of blood vessels and reduced tumor growth *in vivo*[102]. Anti-Dll4 antibodies are now being used in clinical trials.

Natural agents

The empirical screening of thousands of molecules permits identifying compounds with potential antineoplastic effects within the available GBM preclinical experimental models. The Notch signaling pathway is the target of several natural compounds, and although the exerted molecular mechanisms are not completely understood, they have been tested for therapeutic aims. Resveratrol (RSV), a well-known polyphenol[103,104], may reverse multidrug resistance, and several reports demonstrate that it can act as a sensitizer in different types of tumor cells to standard therapeutic agents[105]. Resveratrol inhibited HEY1 levels and NCID in GBM cells. Moreover, we found that low doses of RSV and GSI cotreatment resulted in the induction of GBM cell apoptosis and the concomitant block of the autophagic flux. We showed a prolonged rise of microtubule-associated protein light chain 3 (LC3-II) and p62 expression levels, related to a marked decrease of cyclin-dependent kinase 4 (CDK4), an important regulator of lysosomal function. The activation of autophagy after RSV and GSI stimulus, when the cells have impaired lysosomal function, caused the collapse of the system and thereafter apoptosis. The current studies addressed how the Notch inhibitor and RSV combination could be prospectively implemented in a novel therapeutic strategy for GBM treatment[81]. In A172 and T98G GBM cell lines with a heterozygous p53 mutation, RSV restored wild-type p53 expression, and these effects were mediated by the stimulation of Notch1 levels in a time-

dependent manner. Inhibition of AKT and B-cell lymphoma 2 (BCL-2), increase of BCL2-associated X (BAX) expression, and cleavage of caspase 3 were observed, indicating the great pro-apoptotic effect of RSV[106]. Some *in vitro* and *in vivo* studies have shown that alpinetin, a natural chalcone distributed in a wide range of plants, can suppress the proliferation and invasiveness of GSCs by suppressing Notch signaling[107]. Diosgenin, a natural steroidal sapogenin, induces the differentiation of GBM cells, as shown by the increase of GFAP protein levels and decrease of N-MYC, telomerase reverse transcriptase (TERT), and Notch1[108]. Additionally, falcarindiol, a cytotoxic and anti-inflammatory polyacetylenic oxylipin, has anticancer properties against GBM cells by evoking the differentiation of GSCs, as well as triggering the apoptosis pathway. Conversely, the authors showed that falcarindiol has detrimental effects by disrupting the maintenance of normal neural stem cells and changing the balance between self-renewal and differentiation by suppressing forkhead transcription factor family O (FoxO), which preserves the adult stem cell population through the Notch axis[109].

Other agents

Antihelmintic niclosamide is a previously unrecognized candidate for clinical development. Niclosamide led to cytostatic, cytotoxic, and antimigratory effects, strongly reducing the frequencies of multipotent/self-renewing cells *in vitro* and *in vivo*. Mechanism of action analysis revealed that niclosamide simultaneously inhibited intracellular signaling, including Notch, mTOR, and NF-κB signaling cascades. Additionally, deletion of the nuclear factor kappa-b inhibitor alpha (NFKBIA) locus, encoding the inhibitor of the NF-κB and EGFR signaling pathway, could be a biomarker that predicts the synergistic activity of niclosamide with TMZ for GBM therapy[110]. Several studies consider the idea of peptides inhibiting intracellular Notch signaling. A peptide that mimics MAML1 binding to NICD–RBPJ has been shown to disrupt the ternary transcription complex and pass through the cell membrane[111].

CONCLUSIONS AND FUTURE DIRECTIONS

This review highlights the compelling body of evidence that demonstrates the pivotal role of Notch signaling in coordinating mechanisms of therapy resistance in high-grade brain tumors. The molecular basis of therapeutic resistance in GBM is complex and multifaceted due to the dynamic interplay between the tumor cells, GSCs, TME, and different aberrant signaling, including Notch signalling. Recent studies have evidenced that Notch signaling exerts a dynamic action sustaining the complex interaction among different cell types composing TME, thereby driving GBM recurrence and therapy resistance. Furthermore, redundant signaling pathways converging on Notch signaling can determine increased stem cell population, expression of multidrug transporters, and alteration of DNA repair activity, which control the emergence of disease progression. Besides, the worse prognosis of GBMs and the high levels of radio- and chemoresistance are strictly connected with the highly infiltrative capacities of these tumors, which are partially Notch pathway-dependent. Therefore, the successful treatment of GBM may rely on combinations of therapies, acting on multiple specific targets, converging on Notch inhibition, which could overcome drug resistance. The design of drug dosing schedules and delivery methods to improve brain penetration, together with a better understanding of GBM heterogeneity and Notch orchestrated signaling network, would assure the best possible performance when the treatments are used in combination therapy settings. However, further studies are necessary to ascertain the role of Notch as a predictive biomarker of acquired chemoresistance to be implemented in molecular screening strategies that would be crucial for identifying patients who might benefit from specific treatment combinations.

DECLARATIONS

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Authors' contributions

Conceptualization: De Amicis F
Preparing the manuscript: De Amicis F, D'Amico M
Collecting data:De Amicis F, D'Amico M
Writing, and editing: De Amicis F
Preparing figures: De Amicis F, D'Amico M
Authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Perspectives of metal-organic framework nanosystem to overcome tumor drug resistance

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Abstract

Cancer is one of the most harmful diseases in the world, which causes huge numbers of deaths every year. Many drugs have been developed to treat tumors. However, drug resistance usually develops after a period of time, which greatly weakens the therapeutic effect. Tumor drug resistance is characterized by blocking the action of anticancer drugs, resisting apoptosis and DNA repair, and evading immune recognition. To tackle tumor drug resistance, many engineered drug delivery systems (DDS) have been developed. Metal-organic frameworks (MOFs) are one kind of emerging and promising nanocarriers for DDS with high surface area and abundant active sites that make the functionalization simpler and more efficient. These features enable MOFs to achieve advantages easily towards other materials. In this review, we highlight the main mechanisms of tumor drug resistance and the characteristics of MOFs. The applications and opportunities of MOF-based DDS to overcome tumor drug resistance are also discussed, shedding light on the future development of MOFs to address tumor drug resistance.

Keywords: Metal-organic framework, drug resistance, drug delivery, cancer therapy, nanosystem

INTRODUCTION

Cancer ranks as the second leading cause of death worldwide among various diseases. According to

statistics and estimates by the World Health Organization (WHO), there are 19.3 million new cancer cases and nearly 10 million deaths worldwide in 2020[1]. The global cancer burden is expected to be 28.4 million cases in 2040, although cancer treatment technologies including surgery, chemotherapy[2-5], radiation therapy[6], chemodynamic therapy (CDT)[7,8], gene therapy[9-11], photothermal therapy (PTT)[12], and immunotherapy[13-15] have shown great progress in prolonging survival of cancer patients. However, the multidrug resistance (MDR) of anticancer drugs seriously affects the therapeutic effect, leading to tumor recurrence and metastasis, which has become one of the main obstacles in tumor treatment[16,17].

With the rapid progress of nanotechnology, multifunctional nanoparticles (NPs) enable selective delivery of therapeutic and/or diagnostic drugs to target tumor sites, making precision therapy possible. To date, many organic/inorganic nanomaterials[18-20], including polysaccharides[21,22], proteins[23,24], polymers[25], metals and metal oxides[26,27], mesoporous silica[28], engineering macrophages[29], nucleic acid nanodevices[30-32], and metal-organic frameworks (MOFs)[33,34], have been extensively designed to address physicochemical issues associated with free drugs, tumor drug resistance, and biological barriers during delivery. Among them, MOFs, as an emerging class of nanomaterials, have gained the broad interest of many researchers owing to their high specific surface area, tunable porous structure, satisfactory stability, and biocompatibility. In particular, MOFs have shown great potential and advantages as nanocarriers for drug delivery and protection, such as encapsulation of drugs, direct assembly of drug molecules as organic ligands[35], post synthesis[33], and surface modification[36]. Up to now, the MOF-based drug delivery system (DDS) has been employed to deliver drugs such as cisplatin[37], doxorubicin (DOX)[38-40], biomolecular agents[2,41-43], and immunosuppressants[44,45]. Both single and multiple drug delivery using MOF-based DDS is available[36,46], and combined therapeutic approaches with multiple mechanisms are also commonly performed[47]. In this regard, MOFs, as the carrier in the nanosystem, could protect the drug from degradation, controllably release the drug at the tumor site, inhibit the expression of drug-resistant proteins and genes, and modify the physiological state of the tumor microenvironment, which could enhance the therapeutic effect and mediate the immune behavior against the drug-resistant behavior of cancer cells[44].

To achieve better outcomes in MOF-based nanotechnology for cancer therapy, it is an urgent need to gain a deeper understanding of the mechanisms of tumor resistance and evaluate the potential as well as the challenges of MOF-based DDS in tumor resistance.

MECHANISMS OF TUMOR RESISTANCE

MDR in tumors is complex and multifactorial, including biological barrier formed by the tumor microenvironment (TME)[48-53] and overexpression of drug efflux transporter resulting in the inability to accumulate drugs intracellularly[40,54-57], the drug inactivation due to the specific environment of gene control and metabolism, the resistance to apoptosis and deoxyribonucleic acid (DNA) damage repair[58-63], and immune evasion[64-71], as shown in Figure 1.

Biological barriers

The biological barrier formed by the extracellular matrix (ECM) of the dense tumor site makes it difficult for drugs to reach the target site and is the main reason for human pancreatic ductal adenocarcinoma (PDAC) being one of the least curable and most malignant cancers. PDAC is characterized by the presence of a rich matrix. Pancreatic stellate cells secrete an excess of ECM proteins comprising the matrix with collagen, fibronectin, laminin, and glycoprotein as the main components[51], following the activation by pro-fibrotic mediators including transforming growth factor β (TGF-β). The high density of matrix binds to a large number of stromal cells to form a biological barrier, and the two matrices work together to prevent drug penetration into the cancer cells. Recently, some researchers have developed new strategies to

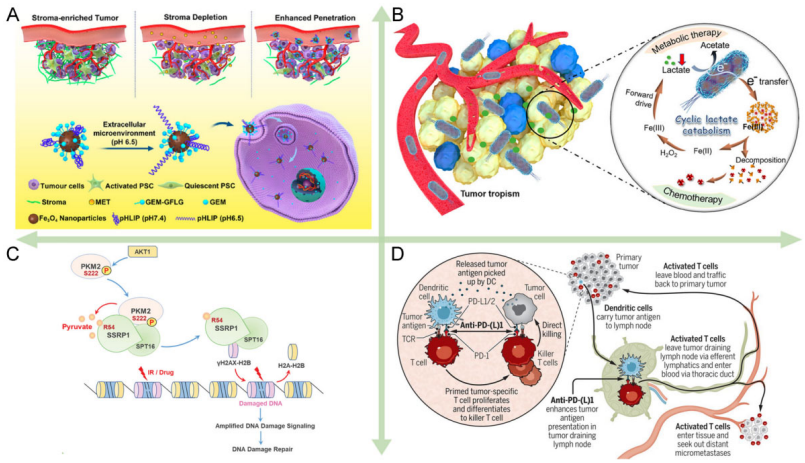

Figure 1. The major mechanisms of tumor resistance. (A) Biological barriers[48]. Reproduced with permission from ref[48]. Copyright 2020 American Chemical Society. (B) Drug inactivation[40]. Reproduced with permission from ref[40]. Copyright 2021 American Chemical Society. (C) DNA damage repair[61]. Reproduced with permission from ref[61]. Copyright 2022 the authors. (D) Immune evasion[69]. Reproduced with permission from ref[69]. Copyright 2020 Science.

overcome the barrier. For example, Han *et al.* utilized metformin to disrupt the dense matrix by activating adenosine phosphate-activated kinase pathway, thereby promoting the penetration and therapeutic effect of the DDS [Figure 1A][48]. Lv *et al.* showed that PDAC cells utilized gasdermin E to mediate resistance to digestive juices in the pancreatic microenvironment[72].

Dense ECM is a distinctive feature of PDAC, but overexpression of drug efflux pumps is more prevalent in more cancer cells. The overexpression of drug efflux pumps is one of the essential mechanisms of MDR in cancer cells[50,52], as various adenosine triphosphate (ATP)-binding cassette transporter family proteins are expressed on the cell membrane. Most of these proteins are ATP-driven multidrug efflux pumps such as MDR protein and P-glycoprotein (P-gp). After the intracellular drug binds to the transporter on the cell membrane, ATP hydrolysis drives a conformational change and pushes the drug out of the cell, resulting in the inability of the intracellular drug to accumulate in sufficient quantities to kill the cells[73]. Ruan *et al.* used nanocarriers to reverse drug resistance induced by overexpression of the drug efflux pumps by inhibiting mitochondrial ATP synthesis[52]. The cyclin-dependent kinase 6-phosphoinositide 3-kinase (CDK6-PI3K) signaling axis is an effective target for attenuating ATP binding cassette subfamily B member 1/P-gp-mediated MDR in cancer cells[11,74,75].

Drug inactivation

The cancer cells are continuously subjected to intense metabolic activity and exhibit hypoxia, high lactate content, and slightly acidic TME. Thereinto, lactate has been shown to be involved in downregulating the expression of the drug efflux pumps P-gp and reducing drug resistance[40], while hypoxia causes the failure of many kinetic treatments that rely on oxygen for oxidative stress. In the drug-induced adverse factors, cancer cells will activate metabolic adaptations to eliminate the adverse factors and evade anticancer treatment [Figure 1B]. Activated metabolic adaptation consists of two major cellular pathways, mitochondrial oxidative phosphorylation (OXPHOS) to glycolytic ATP production and autophagy to recycle harmful substances within the cells[54]. To keep a higher level of metabolism, cancer cells maintain a stable antioxidant defense system internally with a relative balance between glutathione (GSH) and reactive oxygen species (ROS)[55]. Drugs entering the cells are often depleted by GSH and cannot increase ROS content to generate oxidative stress, which leads to the failure of dynamic therapy. Resistance based on GSH and ROS mechanisms has led to the inefficiency or even failure of most chemotherapeutic and kinetic

treatments that rely on stimulating oxidative stress[3,56,57,76].

Wang *et al.* demonstrated that nitric oxide (NO) could be used to reduce oxygen consumption by inhibiting cancer cell respiration, thereby eliminating hypoxia-induced chemoresistance of DOX[56]. Jiang *et al.* synthesized photosynthetic microcapsules to successfully complete sustained photosynthetic oxygenation in cancer cells, inducing lipid peroxidation and iron death processes to kill cancer cells[76].

The emergence of secondary drug resistance has exacerbated the difficulty of treating cancer with some drugs. Secondary drug resistance indicates that, after being treated with one drug, cancer cells become resistant to another specific drug that was not previously used[77]; that is, its development is the dynamic clonal evolution of primary resistance to the drugs used. Aldonza *et al.* found that cancer cells with primary acquired resistance to the microtubule-stabilizing drug paclitaxel also tended to exhibit resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), even though the cancer cells had never been exposed to this drug before[77].

Resistance to apoptosis and DNA damage repair

Cancer cells, which are generated from gene mutations in normal cells, can autonomously control the apoptotic program and enter an endless cell cycle. To induce DNA damage in cancer cells to cause cell necrosis and apoptosis is an overwhelming chemotherapeutic idea to destroy cancer cells. The chemotherapeutic drug cisplatin interacts with DNA to inhibit DNA replication and induce apoptosis killing cells. The chemotherapeutic drug DOX, a topoisomerase II (TOP2) inhibitor, inhibits DNA remodeling by inserting into double-stranded DNA and suppressing TOP2 activity. However, DNA damage repair (DDR) plays an important role in resistance to apoptosis mediated by anticancer drugs, including base excision repair, base mismatch repair, nucleotide excision repair, and DNA double-strand break repair[78]. Proactively enhanced DDR produces resistance of cancer cells to DNA damage-based therapies. Recently, Wu *et al.* observed that the products of glycolysis and exogenous pyruvate in cancer cells could enhance H2A variant H2AX serine (S) 139 (γH2AX) loading on chromatin, thereby promoting DNA repair [Figure 1C][61]. Chen *et al.* employed a hypoxia-activated chemotherapeutic in cancer cells to downregulate the DNA repair protein, xeroderma pigmentosum group F, whose overexpression triggered resistance to cisplatin treatment[3].

Cancer cells could prevent apoptosis through immune checkpoint blockade and autophagy[58,60,79]. Among them, immune checkpoint blockade tends to target extracellular immune cells for killing, while apoptosis is evaded intracellularly by autophagy and regulation of partial protein expression[50]. Cancer cells can suppress apoptosis by the overexpression of anti-apoptotic proteins (e.g., B-cell lymphoma-2 (BCL-2), BCL-XL, and myeloid cell leukemia-1 (MCL-1)) to downregulate or inactivate pro-apoptotic proteins (BCL-2-associated X, BCL-2-associated K, and BCL-2-related ovarian killer)[59]. Chen *et al.* used synthetic NPs to destabilize microtubules and prevent the formation of spindles in normal mitosis, leading to abnormal cell division and eventually apoptosis[62]. Wang *et al.* found that acetaldehyde dehydrogenase (ALDH2) gene was involved in mediating the RAF/MAPK signaling pathway, which is engaged in the regulation of apoptosis and drug sensitivity[74].

Immune evasion

Cancer immunotherapy has achieved clinical success and the use of T cells to kill cancer cells is a highly effective therapeutic mechanism. However, cunning cancer cells can evade the immune response by shaping immunosuppressive TME and immune checkpoint blockade. Joung *et al.* revealed that four genes, programmed death-ligand 1 (PD-L1), MCL-1, JunB proto-oncogene (*JUNB*), and β1,3-N-acetylglucosaminyltransferases (*B3GNT2*), affect the interaction of cancer cells with T cells, conferring

resistance to T cell cytotoxicity[64]. Programmed cell death protein 1 (PD-1) on the T cells interacts with PD-L1 on the cancer cells to inhibit activation of T cells[67]. To address this problem, Topalian *et al.* proposed neoadjuvant PD-(L)1 blockade therapy to enhance systemic immunity against tumors and prevent recurrence [Figure 1D][69]. In addition, Huang *et al.* summarized that multiple checkpoint blockades, including PD-1 and cytotoxic T lymphocyte antigen-4 (CTLA-4), developed an immunosuppressive TME in melanoma tumors[65]. Recently, Saha *et al.* revealed that cancer cells can interact with platelets entering TME to produce chemoresistance[80]. The platelet has been increasingly found to work as an important activator to induce epithelial-mesenchymal transition (EMT), while EMT is the primary and key event in promoting the distant spreading of metastatic tumor cells[81]. In another study, indoleamine 2,3-dioxygenase (IDO) in TEM was found to catalyze the metabolism of L-tryptophan to L-kynurenine, thus inhibiting the increase of effector T cells and promoting the growth of regulatory T (Treg) cells[70].

METAL-ORGANIC FRAMEWORK NANOSYSTEM

Synthesis of MOFs and drug loading methods

MOFs are a class of crystalline porous materials with periodic network structures constructed by metal (cluster) nodes and organic ligands through coordination self-assembly[82]. Benefiting from the abundant species of metal (cluster) nodes and organic ligands, and the diverse coordination patterns between them, tens of thousands of MOFs have been reported. The research on MOFs in therapy has exhibited rapid growth in recent years [Figure 2].

Various synthetic methods provide flexibility in the synthesis of MOFs. Solvothermal and non-solvothermal methods are common approaches for the fabrication of MOFs. Solvothermal synthesis is typically performed at high temperatures or high pressure to dissolve the reagents and facilitate the synthesis. Non-solvothermal synthesis is performed at temperatures that are lower than the boiling point of the solvent and favorable for nucleation. Non-traditional synthetic approaches for drug delivery include microwave, sonication, mechanical milling, or electrochemical synthesis[83].

The key challenge for the application of MOFs in the biomedical field lies not only in the precise control of their synthesis but also in the effect of surface affinity on their application behavior. Remarkably, the attractiveness of MOFs as drug delivery vehicles is mainly due to their special drug-carrying capacity. The drug loading efficiency is determined by the physical properties of the MOF (e.g., pore size, surface area, and spatial structures). The loading methods are essential to maximize loading and attain the ideal release. There are three common drug loading techniques for MOFs: one-pot synthesis[84], biomimetic mineralization[85], and post-synthesis loading[86] [Figure 3].

One-pot synthesis is the co-precipitation of therapeutic molecules with MOFs during synthesis, resulting in a uniform distribution of drug agents in the pores of the MOF. One-pot synthesis is beneficial for the controlled release of drugs in the MOF, provided that the pore size of the MOF is small enough to limit the rapid diffusion of drugs through the MOF structure [Figure 3A][84]. Biomimetic mineralization is valuable for loading biological agents such as protein and nucleic acid. Unlike one-pot synthesis, biomimetic mineralization utilizes bioagents as nucleation sites for MOF crystallization [Figure 3B][85]. Specifically, the biomolecule partially interacts with the MOF building blocks, thereby promoting nucleation. Thus, the encapsulated biological molecules determine the sizes, morphologies, and crystallinities of the MOFs. Such an efficient encapsulation mechanism has been proved to protect bioagents from harsh chemical environments, heat, and degrading enzymes. Since the therapeutic drugs are integrated into the MOF, their release is dependent on the degradation of the MOF, which may lead to the sustained release and delayed activity of the encapsulated drugs. Post-synthesis loading involves the encapsulation of medicaments into

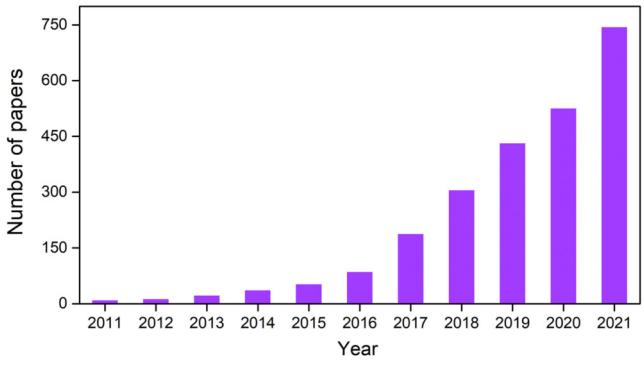

Figure 2. The number of publications in the last decade on the topic of therapy using MOFs according to Web of Science search.

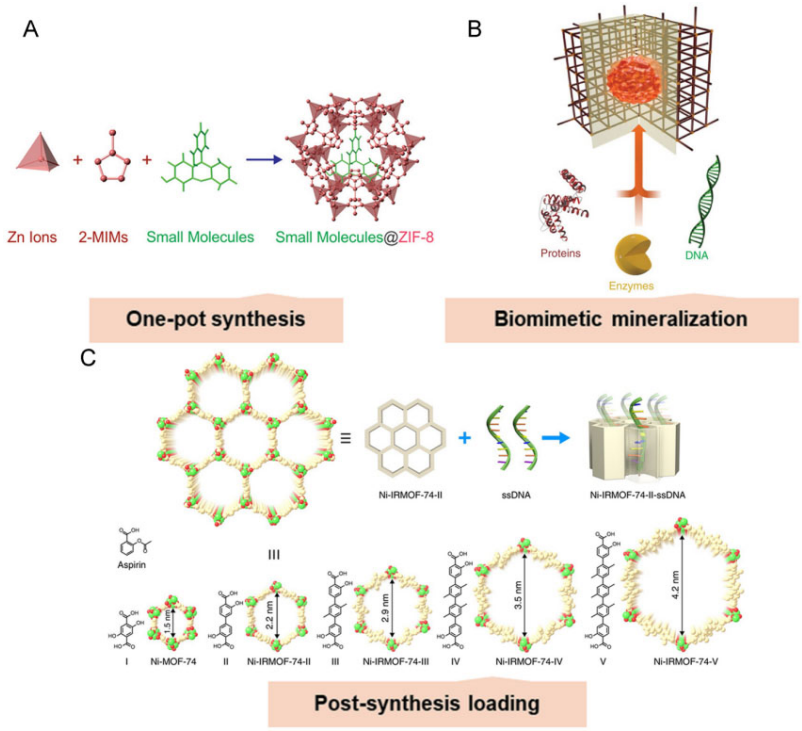

Figure 3. The common drug loading methods on the MOFs. (A) One-pot synthesis[84]. Reproduced with permission from ref[84]. Copyright 2014 American Chemical Society. (B) Biomimetic mineralization[85]. Reproduced with permission from ref[85]. Copyright 2015 the authors. (C) Post-synthetic loading[86]. Reproduced with permission from ref[86]. Copyright 2018 the authors.

the pores or the surface of the MOF after synthesis [Figure 3C]. This could be obtained commonly by mixing the MOF and medicaments in a solvent, followed by evaporation to remove the solvent[86,87]. Surface loading is typically dominated by unsaturated metal site interactions and electrostatic interactions and could also be implemented by attaching the drugs to the polymer-coated surface[88]. Unfortunately, surface loading normally leads to reduced drug loading in contrast to other approaches. Overall, the high porosity of the MOF provides space for the loading of guest molecules. The well-defined structure of MOFs has a clear relationship to their properties, which provides guidance for future modifications. Regardless of the encapsulation method employed, MOFs protect bioagents from degradation and expand potential avenues for clinical drug delivery.

Advantages of MOFs

MOFs have not only excellent porous properties (e.g., high specific surface area and high porosity) but also many advantages such as structure design and function adjustment, so they have been widely applied in gas adsorption and separation[89,90], chemical sensing[91,92], heterogeneous phase catalysis, biological diagnosis[93,94], and cancer therapy. In view of the flexibility in the design and synthesis of MOF materials, it is convenient to introduce primitives into MOFs through metal nodes, ligands, and guests to endow such materials with unique activities, thereby constructing multifunctional MOFs. Different from the simple loading of primitives by traditional heterogeneous porous materials, the structure of MOFs is ordered and observable, which is conducive to understanding the distribution of primitives in the framework and their interactions at the microscopic scale. Therefore, the structure-activity relationship between spatial structure and performance could be better understood. Furthermore, MOFs are tunable in structure and easily modified. It is easy to adjust the structure of MOFs and introduce various functional groups through crystal engineering and to exert a synergistic effect among the active centers through a specific spatial arrangement in MOFs, thereby regulating chemical interactions between multiple components within the framework. In addition, the rich and diverse pore structures of MOFs are not only conducive to material transport, but also can mimic the function of enzymes. Their unique cavities can generate a "spatial confinement effect" on the guest molecules, thereby influencing the behavior between the host and the guest from a kinetic aspect. Therefore, MOFs, as a class of excellent nanocarriers, are characterized by porosity, heterogeneous properties, and biocompatibility, which endow them with unique advantages in the fields of enzyme-like catalysis, disease diagnosis[95], therapy[96,97], and bioimaging.

Potential biomedical application of MOFs

Drug delivery

To overcome the inherent limitations of therapeutic drugs and achieve targeted delivery and controllable release of drugs, the development of drug nanocarriers has become a research hotspot. One of the most essential considerations for drug nanocarriers is that the drugs must be released at a certain rate until the target site is reached, achieving the appropriate dose in a given time. The biological metal-organic framework (BioMOF), connecting MOF chemistry with bioscience, has become a drug delivery vehicle due to its high drug loading capacity and excellent biodegradability[98,99]. Recently, Ni *et al.* synthesized MIL-100 NPs loaded with chemotherapeutic drug mitoxantrone and hyaluronic acid (HA) [Figure 4A][93]. The NPs targeted cancer cells with recognition of cluster of differentiation 44 (CD44) by HA, while co-injected anti-OX40 antibody (αOX40) reversed the immunosuppressive effect, allowing NPs to enter cancer cells favorably and release drug for chemotherapy. A bimetallic MOF for intracellular drug synthesis and self-sufficient therapy was designed [Figure 4B][42]. Copper ions that were liberated from MOFs could catalyze the drug synthesis, killing tumors on site to minimize side effects on normal cells.

Biological imaging

The rapid development of bioimaging technology has contributed significantly to exploring the pathological characteristics and metabolic functions of biological tissues by providing vital equipment, which has greatly facilitated the diagnosis of diseases. More recently, MOF-based nanocomposites have been widely used in fluorescence imaging (FL), computed tomography (CT), and magnetic resonance imaging (MRI), as well as other fields, due to their simple functionalization, diverse structures and compositions, and large porosity[100,101]. Cheng *et al.* prepared a bimetallic Cu/Zn-MOF, further hollowed the multivalent ($Cu^{+/2+}$/$Mn^{2+/4+}$) structure after heating in manganese(II) acetylacetonate, and finally loaded indocyanine green (ICG). This MOF nanocarrier released Mn^{2+} for achieving MRI via a Fenton-like reaction and ICG for turn-on FL[101]. Li *et al.* synthesized a covalent organic framework cladding MOF (MOF@COF) encapsulated with Bi^{3+}, Mn^{2+}, and meso-Tetra (4-carboxyphenyl) porphyrin (TCPP) [Figure 4C][102]. Upon reaching the tumor site, the nanocapsules degraded and Bi could be used for CT while TCPP was used for FL, ensuring

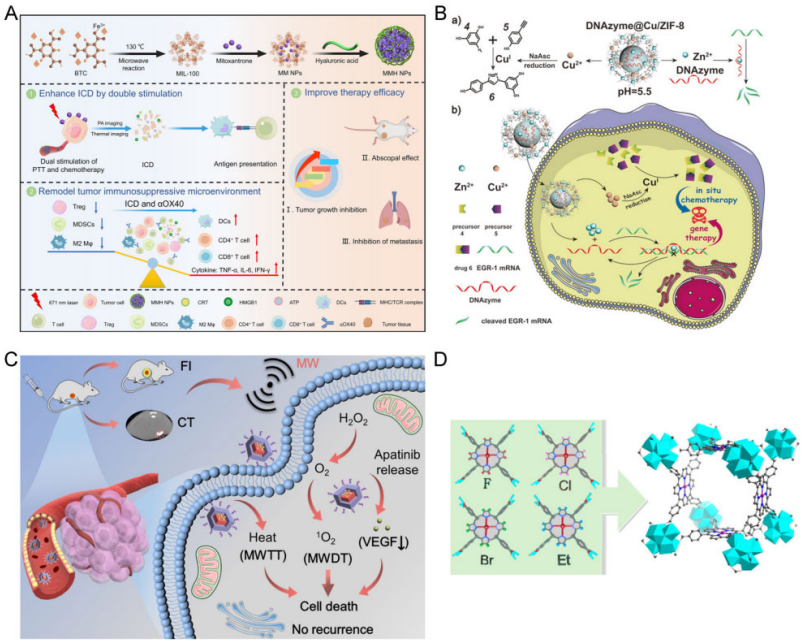

Figure 4. Summary of the major junctions of MOFs in biomedical application. (A) Drug delivery[96]. Reproduced with permission from ref[96]. Copyright 2021 American Chemical Society. (B) Intrinsic therapeutic MOF[42]. Reproduced with permission from ref[42]. Copyright 2021 Wiley-VCH. (C) Biological imaging[102]. MWTT, microwave thermal therapy; MWDT, microwave dynamic therapy; VEGF, vascular endothelial growth factor. Reproduced with permission from ref[102]. Copyright 2022 Elsevier. (D) Biomimetic catalysis[105]. Reproduced with permission from ref[105]. Copyright 2017 American Chemical Society.

extremely high imaging performance.

Biomimetic catalysis

An enzyme is a robust macromolecular biocatalyst that accelerates chemical reactions with unparalleled efficiency and specific selectivity under mild conditions. Thus, biomimetic catalysis for tumor therapeutic application is a highly attractive field with broad prospects for efficient catalysis[103]. The construction of functional systems that mimic natural enzymes is a remarkably popular goal. Especially, metalloporphyrins and their derivatives have been attracting attention as the catalytic centers of certain enzyme families. Feng *et al.* reviewed the precise synthesis of various MOFs using robust and multifunctional porphyrins as ligands and evaluated the efficient enzyme-like catalytic ability of porphyrin MOFs exhibited in oxidation catalysis, Lewis acid catalysis, electrocatalysis, and photocatalysis, providing ideas for the future synthesis of specific functional porphyrin MOFs and the development of their enzyme-like catalytic function[104]. Zr-MOF-based single substitution toward high-performance catalysis was proposed, achieving the molecular-level control of the chemical environment around the catalytic center [Figure 4D][105]. Zhao *et al.* demonstrated the synthesis of Corrole-based MOF exhibits more efficient heterogeneous catalytic action than the porphyrin-based MOFs[106].

THERAPEUTIC APPLICATIONS OF MOF NANOSYSTEM TO OVERCOME TUMOR RESISTANCE

Improving delivery

Normally, some drugs are consumed in the blood circulation by autoimmune system before they come to the tumor. The barriers from TME and cancer cells could work together to prevent drugs from entering the cells and even excrete drugs that have entered cells due to the overexpression of the drug efflux pump,

making it difficult for the drugs to accumulate effectively in the cell. MOF-based NPs are allowed to accumulate in the tumor through enhanced permeability and retention effects (EPR)[107]. For example, Chen *et al.* developed an HA-coated Zr(IV)-based porphyrinic MOF DDS loaded with α-cyano-4-hydroxycinnamate (CHC) [35]. This DDS was delivered to cancer cells with CD44 overexpression owing to the targeting ability of HA towards CD44, and then released Zr (IV)-based porphyrinic MOF and CHC for enhanced photodynamic therapy (PDT) effect. Du *et al.* synthesized DOX@NH_2-MIL-88B-COD@CS NPs, which were loaded with cholesterol oxidase (COD), DOX, and chondroitin sulfate (CS) gel shell[39]. The COD catalyzed the degradation of cholesterol on the cell membrane to weaken the bio-barrier of cell membrane, thus facilitating the delivery of NPs into the cell. Meanwhile, H_2O_2 generated from the cholesterol degradation was catalyzed by the nanoenzyme, NH_2-MIL-88B (MIL, Materials of Institute Lavoisier), to produce •OH to kill cancer cells. An erythrocyte membrane camouflaged iron-based MOF was developed for the multidrug delivery platform [Figure 5A][55]. Erythrocyte membrane prolonged the circulation cycle of the multidrug platform in the blood as well as provided targeting. After endocytosis and catabolism, the multidrug platform released iron and multiple drugs, triggering the ferroptosis process and chemotherapeutic effect towards tumors. In addition, Cheng *et al.* utilized cell membrane artefacts from human breast cancer MDA-MB-231 and MOF to protect gelonin from catabolism[108]. The gelonin@ZIF-8/MDA-MB-231 (ZIF, zeolitic imidazolate framework) cell membrane was 11-fold more effective than free gelatin in cancer treatment. The biomimetic cascade MOF nanoreactor loaded with glucose oxidase (GOD) and DOX for starvation-amplified chemotherapy combination therapy demonstrated powerful anticancer efficacy[109].

The efflux of intracellular drugs has been a difficult obstacle in the fight against MDR due to the overexpression of ATP-binding cassette transporter family proteins P-gp on cancer cell membranes. MOF-based NPs could enter the cell by endocytosis and ensure their release in the perinuclear region, bypassing membrane transporters. Innovating upon this evidence, Wang *et al.* showed a biohybrid bioreactor through the integration of DOX-loaded MIL-101 and a *Shewanella oneidensis* (SO) bacterium[40]. After injection, the bioreactor migrated to the tumor site with high lactic acid content. The metabolism of lactic acid in TME led to the degradation of MIL-101, the release of DOX, and the downregulation of P-gp, which was related to the tumor multidrug resistance, improving the therapeutic efficiency.

Overcoming obstacles

The specific TME is resistant to the delivery and action of drugs[48,110,111]. PDAC has the highest mortality rate among various types of cancers owing to the high density of matrix which prevents drug penetration into the cancer cells[48]. Not only hypoxic environments but antioxidant defense systems mediate the resistance of PDT[110]. Some signaling, such as S100A9-CXCL12, creates a TME, rendering cancers insensitive to immunotherapy[111]. Even under high metabolic intensity, cancer cells tend to adopt the metabolism of glycolysis that consumed large amounts of oxygen and produced large amounts of lactate, resulting in a hypoxic state. The metabolite lactate promotes the development of drug resistance [Figure 5B][40]. The hypoxic also greatly reduces the efficacy of kinetic therapies which rely on the conversion of ROS to stimulate oxidative stress. Intracellular autophagy and high levels of GSH lead to rapid degradation of drugs or resist the process of drug-induced cell death. Researchers have reversed resistance to enhanced therapy by increasing oxygen or ROS levels and depleting GSH. Since the drugs are susceptible to degradation, their activities could be maintained for a long time by the protection of a biocompatible and chemically stable MOF. We summarize the recent MOF-based materials to overcome cancer drug resistance in Table 1. For example, Lian *et al.* used PCN-333(Al) (PCN, porous coordination network) as the nanocarrier to load tyrosinase (TYR) for synthesizing an enzyme-MOF nanoreactor, converting the non-toxic prodrug paracetamol (APAP) to 4-acetamido-o-benzoquinone (AOBQ) *in vivo*[33]. They demonstrated that AOBQ induced the depletion of GSH and oxidative stress, attracting ROS-generated CDT to poison cancer cells.

Table 1. A summary of MOFs to overcome cancer drug resistance

MOFs	Drugs	Cancer treatment technologies	Methods of overcoming resistance	Reference
Cu/ZIF-8	DNAzym, Cu^{2+}	Gene therapy, chemotherapy	Drug intracellular activation, precise target	Li *et al.*[43]
CuTPyP	DOX, porphyrin	Chemotherapy, PDT	Efficient intracellular drug accumulation, cellular resensitization	Jiang *et al.*[4]
MIL (Fe)	DOX, Fe	Chemotherapy, CDT	Intracellular drug efflux inhibition, GSH consumption	Peng *et al.*[55]
MIL-101	DOX	Chemotherapy	Iintracellular drug efflux inhibition	Wang *et al.*[40]
MnMOFs	Cisplatin, Mn	Chemotherapy, microwave thermotherapy	GSH consumption, increasing of ROS levels	Wu *et al.*[37]
MOF-199	GOD, DQ	Chemotherapy, CDT	•OH generation, amplified effect	Pan *et al.*[7]
MOF-199	IDO, NO	Immunotherapy	Increasing of T cell infiltration	Du *et al.*[44]
NH2-MIL-88B	DOX, COD	Chemotherapy, CDT	Enhanced sensitivity of tumor cells to drug	Du *et al.*[39]
PCN-333 (Al)	TYR, AOBQ	Chemotherapy, CDT	*In vivo* enzymatic synthesis of drugs, precise target	Lian *et al.*[33]
Se/Ru-decorated porous MIL-101	siRNA	Gene therapy	Promoted siRNA escape from endosomes/lysosome	Chen *et al.*[62]
UiO-66-NH_2	Cisplatin, DOX	Combination chemotherapy	Prolonged circulation process, efficient intratumoral accumulation	Hu *et al.*[36]
ZIF-8	Immunogenic dead cancer cells	Immunotherapy	Enhanced immunogenicity	Yang *et al.*[45]
ZIF-8	CQ, GOD	Starvation therapy	The autophagy inhibition	Li *et al.*[47]
ZIF-8	DOX, GOD	Starvation therapy, chemotherapy	TME adjustment, drug intracellular activation, precise target	Cheng *et al.*[109]
ZnMOF	DOX, quercetin	Chemotherapy	Efficient intracellular drug accumulation	Sun *et al.*[38]
Zr(IV)-based porphyrinic MOF	CHC	PDT	Reduced cellular O_2 consumption, relieve hypoxia	Chen *et al.*[35]

DOX: doxorubicin; PDT: photodynamic therapy; CDT: chemodynamic therapy; GSH: glutathione; ROS: reactive oxygen species; AOBQ: 4-acetamido-o-benzoquinone.

The cytotoxicity of enzyme from the enzyme-MOF nanoreactor was still observed after three days, while the free enzyme without MOF protection was completely inactive within a few hours. Pan *et al.* proposed a copper(II) nano-MOF loaded with disulfiram prodrug (DQ) and conjugated with GOD, displaying cascade oxidation and Fenton-like reaction[7]. The nano-MOF was first metabolized by GOD-catalyzed glucose to produce H_2O_2, which triggered the activation of the prodrug DQ to produce highly cytotoxic copper (II) diethyldithiocarbamate ($Cu(DTC)_2$) *in situ* and Fenton-like reaction to generate •OH against cancer.

Polypharmacy and combination therapy

Single drugs and simple single-mechanism therapeutic effects are more likely to produce resistance in MDR. Researchers have found that unexpected efficacy can be achieved when a combination of multiple drugs acts simultaneously in the tumor[112,113]. Combination therapies with multi-drug synergistic effects or different mechanisms in tandem are becoming an effective way to improve tumor therapy. MOFs also show good applicability in constructing multidrug nanosystems. For example, Hu *et al.* synthesized hybrid NPs by employing porous UiO-66-NH_2 (UiO, University of Oslo) as the nanocarrier and polymer as the shell to encapsulate cisplatin in MOF pores and DOX in the polymer shell, which showed high multidrug loading capacity and good biocompatibility [Figure 5C][36]. The negatively charged shell was degraded in acidic TME and the positively charged MOF was then exposed to interact with the negatively charged cell membrane to promote drug intracellularization, lysosomal escape, and nuclear localization. This drug co-loaded hybrid nanosystem showed excellent anti-tumor effects and higher biosafety than the free drugs *in vivo*. In addition, Ling *et al.* synthesized a dual drug-loaded nanosystem in MOF with DOX and 5-fluorouracil,

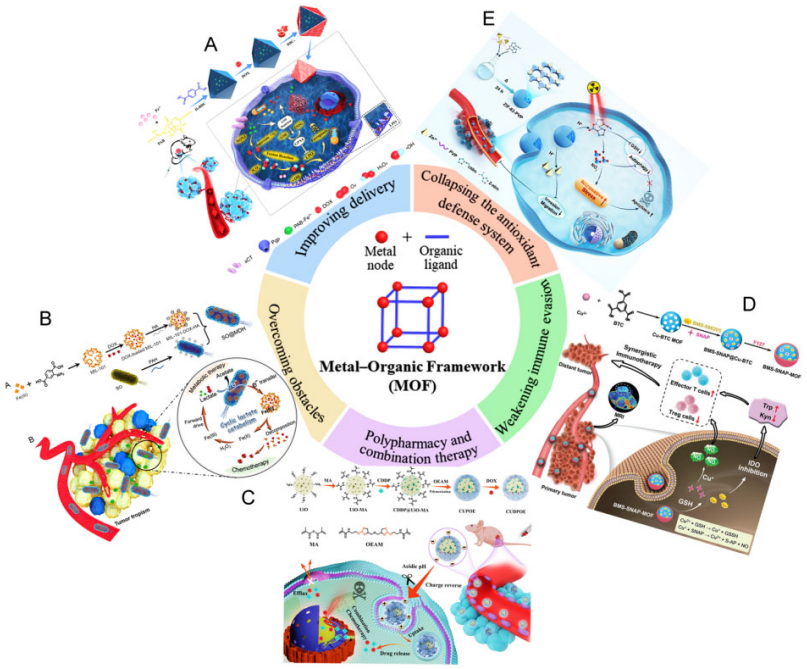

Figure 5. Summary of the therapeutic application of MOFs to overcome tumor resistance. (A) Improving delivery[55]. Reproduced with permission from ref[55]. Copyright 2021 the authors. (B) Overcoming obstacles[40]. Reproduced with permission from ref[40]. Copyright 2021 American Chemical Society. (C) Polypharmacy and combination therapy[36]. Reproduced with permission from ref[36]. Copyright 2021 Elsevier. (D) Weakening immune evasion[44]. Reproduced with permission from ref[44]. Copyright 2022 Wiley-VCH. (E) Collapsing the antioxidant defense system[118]. Reproduced with permission from ref[118]. Copyright 2021 Wiley-VCH.

achieving good results in both anticancer therapy and bioimaging[114]. Jiang *et al.* synthesized a DOX-loaded MOF with porphyrin derivatives as organic ligands, combining chemotherapy with PDT[4]. DOX was shown to enhance the efficacy of PDT, causing more severe mitochondrial membrane potential damage and enhanced inhibition of P-gp.

Weakening immune evasion

Immunotherapy is based on the patients immune systems[115,116]. By weakening the immune evasion of cancer cells, immunotherapy helps the immune system recognize tumors in the body and activate the immune program to clear cancer cells[117]. Immunotherapy has always been the most effective way to treat cancer with few side effects.

IDO plays a key role in mediating the immune evasion of cancer cells. The amino acid L-tryptophan (Trp) can be degraded to L-kynurenine (Kyn) catalyzed by IDO, which has a suppressive effect on cytotoxic T lymphocytes (CTL) as well as activates Treg cells to protect cancer cells. Du *et al.* synthesized a Cu-MOF to load with NO donor s-nitrosothiol groups (SNAP) and IDO inhibitor [Figure 5D][44]. The abundant GSH triggered a cascade reaction to release drugs and produced NO *in situ*. These two substrates synergistically modulated the immunosuppressive TME accompanied with increasing $CD8^+$ T cells, decreasing Treg cells, and weakening the immune evasion of the tumor. This immunotherapy showed significant anticancer effects. Safe, biocompatible, and physiologically stable MOFs show great potential as carriers of genetic material. For example, Chen *et al.* synthesized MIL-101(Fe) encapsulated with specific small interfering ribonucleic acids (siRNAs) and modified with selenium (Se)/ruthenium (Ru) NPs to protect siRNAs from nuclease degradation during targeted delivery into cancer cells[62]. The developed NPs promote cellular uptake and lysosomal escape of siRNA to silence the MDR gene. Previous kinetic therapies mainly killed

cancer cells by elevating intracellular ROS to trigger oxidative stress and collapse the antioxidant defense system, as shown in Figure 5. The hypoxic environment is highly resistant to kinetic treatments such as PDT and CDT. However, hypoxia is ineffective against reactive nitrogen species (RNS) mechanisms as it does not require ROS involvement. Therefore, Li *et al.* utilized 2-nitroimidazole (2-nIm) and 1H-imidazole-4-cyano as ligands and Zn^{2+} as a metal node to synthesize a MOF. 2-nIm produced RNS to damage cancer cells under X-ray by exploiting the RNS mechanism, bypassing the resistance of the hypoxic environment [Figure 5E][118].

FUTURE PERSPECTIVES

MOFs, formed by the coordination between metal ions or metal clusters and organic ligands, have become high-profile nanomaterials in medical research due to their controllable, regular structure, a large number of active sites, tumor microenvironment responsiveness, high biocompatibility, and physiological stability. Many researchers have devoted themselves to developing MOF-based DDS as nanocarriers of anticancer drugs. Although great progress has been made in related research, there are still many difficulties and challenges in the continuous development and translation of this emerging technology into clinical practice. First, the weak interactions between MOFs and drugs limit their development. Using MOFs as a nanocarrier to encapsulate or load anticancer drugs requires strong interactions between MOFs and anticancer drugs, such as the binding of special functional groups (carboxyl groups, aldehyde groups, etc.) and electrostatic adsorption caused by opposite charges. The weak interactions of MOF may result in drug leakage with reduced therapeutic efficiency. Second, the approaches to drug encapsulation into MOFs are limited. At present, there are three methods for the encapsulation of drugs and MOFs: one-pot synthesis, *in situ* encapsulation, and post-synthesis loading. *In situ* encapsulation is drug encapsulation during the synthesis of MOFs, which requires milder MOF synthesis conditions. However, not all MOF synthesis conditions allow *in situ* encapsulation, which limits the choice of carriers and drugs for DDS. Third, biocompatibility and safety issues of MOFs *in vivo* are uncertain. Although the biocompatibility of MOFs has been verified, a comprehensive evaluation of the *in vivo* toxicity of the DDS throughout the treatment process is still required before human therapy. Moreover, in fact, the function of MOFs as a nanocarrier is often to protect and release the drug in MOF-based DDS, while the functions such as targeting the tumor still need to rely on specific modifications.

In future studies, the development and practical application of MOF-based DDS need to overcome the above difficulties, design multifunctional MOFs, design mild synthesis conditions, construct novel MOFs with strong interrelationships between drugs, and evaluate the effects of DDS on organisms throughout the therapeutic process.

CONCLUSION

The research progress of MOF-based anticancer DDS to overcome tumor drug resistance is summarized and reviewed. Among the tumor drug resistance features, biological barrier, drug inactivation, anti-apoptosis, DNA repair, and immune evasion, the tumor microenvironment with multiple resistance mechanisms cannot be ignored, which has a great impact on the drug resistance of tumors. Next, the synthesis and drug loading method of MOFs are described, which is crucial for the excellent drug loading and protection ability of MOFs. The advantages of MOFs and their application potential in drug delivery, bioimaging, and biomimetic catalysis are also demonstrated. Finally, the application of MOF-based DDS in chemotherapy, radiotherapy, immunotherapy, gene therapy, and starvation therapy, as well as combination therapy with various mechanisms, is systematically reviewed from multiple perspectives. The approaches of MOFs to protect drugs, overcome drug resistance, and improve the effectiveness of cancer treatment are expounded from four perspectives: improving drug entry into cancer cells, overcoming drug resistance

barriers, multidrug loading and combination therapy, and reactivating immunotherapy. Compared with other nanocarriers, the tunable structure of MOFs provides great convenience for loading different drugs. The responsiveness of the tumor microenvironment provides a guarantee for drug protection and controlled release, which improves the efficiency of drug utilization. Using MOFs as nanocarriers to load drugs and overcome tumor resistance will become an emerging and achievable technology.

Benefiting from the controllable structure and high porosity of MOFs, based on the known drug resistance mechanisms, we can utilize biological and chemical techniques to load drugs on MOFs, and then modify them to synthesize composites for suppressing drug resistance of cancer cells and improving the efficacy of cancer therapy. Although there is still a certain gap from the actual human treatment and more intensive research is needed, we believe that MOFs will eventually overcome tumor drug resistance and show their great potential to benefit human cancer therapy.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study: Wang H, Wei T
Review the literature: Wang H, Li S, Yang Y, Zhang L, Zhang Y
Wrote the manuscript: Wang H, Li S, Wei T
Finalized the manuscript: Wang H, Wei T
Supervised the manuscript: Wei T

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Perspective

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Loss of HER2 in breast cancer: biological mechanisms and technical pitfalls

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Abstract

Loss of HER2 in previously HER2-positive breast tumors is not rare, occurring in up to 50% of breast cancers; however, clinical research and practice underestimate this issue. Many studies have reported the loss of HER2 after neoadjuvant therapy and at metastatic relapse and identified clinicopathological variables more frequently associated with this event. Nevertheless, the biological mechanisms underlying HER2 loss are still poorly understood. HER2 downregulation, intratumoral heterogeneity, clonal selection, and true subtype switch have been suggested as potential causes of HER2 loss, but translational studies specifically investigating the biology behind HER2 loss are virtually absent. On the other side, technical pitfalls may justify HER2 loss in some of these samples. The best treatment strategy for patients with HER2 loss is currently unknown. Considering the prevalence of this phenomenon and its apparent correlation with worse outcomes, we believe that correlative studies specifically addressing HER2 loss are warranted.

Keywords: HER2 loss, breast cancer, subtype switch, tumor heterogeneity, clonal selection, HER2 downregulation, technical pitfalls

INTRODUCTION

Human epidermal growth factor receptor-2 (HER2)-positive breast cancer (BC) accounts for 15%-20% of breast carcinomas[1]. Although originally associated with a poor prognosis, the advent of anti-HER2 agents has dramatically changed the natural history of HER2-positive BC. Most patients with early-stage disease are now cured following (neo)adjuvant therapy[2,3], and many patients with advanced disease live many years after diagnosis[4,5]. This unprecedented success is due to the development of numerous highly effective HER2-targeting agents capable of inhibiting the HER2 signaling pathway, which is the main driver of cancer cell proliferation and progression for this breast cancer subtype[6]. Indeed, HER2 status is not only a prognostic biomarker but also a strong predictor of response to HER2-targeted therapies[6,7].

According to 2018 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines, HER2 status is assessed by both immunohistochemistry (IHC) and *in situ* hybridization (ISH)[8]. The former describes the expression level of the HER2 protein on the tumor cell membrane, while the latter reflects the amplification of the *ERBB2* gene at the nuclear level. The results of IHC and ISH are consistent in most BCs. However, some cases may instead be "borderline" or equivocal, and they are codified in specific groups by ASCO/CAP guidelines[8].

Discordance between HER2 status in paired samples has been described previously in both early and advanced setting[9,10]. Specifically, the loss of HER2 is defined as a negative HER2 status in a previously HER2-positive tumor (i.e., a change from a 3+ IHC score or 2+/ISH-amplified to an IHC 0-1+ or 2+/ISH-negative), implying that the residual tumor no longer has HER2 overexpression or amplification at surgery, after neoadjuvant treatment[11]. This phenomenon is of particular interest in the landscape of breast cancer care because of its potential impact on treatment choice and prognosis. Although it has been reported in many studies, the reasons behind HER2 loss are still unclear. Both true biological changes and technical pitfalls have been assumed as potential causes of this phenomenon[12] [Figure 1].

BIOLOGICAL MECHANISMS DRIVING HER2 LOSS

Several biological mechanisms may be potentially responsible for HER2 loss, including HER2 downregulation, subtype switching, and clonal selection.

HER2 downregulation

HER2 downregulation - defined as a reduction in HER2 expression at the proteomic level while retaining the *ERBB2* gene amplification - can be induced by anti-HER2 agents acting on the HER2 pathway itself[14]. In untreated HER2-positive tumors, cancer growth is driven by *HER2* gene amplification, which increases transmembrane HER2 tyrosine kinase (TK) receptor expression by up to 100-fold[15]. HER2 overexpression thus induces both homo- and hetero-dimerization of HER receptors, i.e., HER1, HER2, HER3, and HER4; phosphorylation of the kinase domains; and consequent activation of a downstream signaling pathway involving both the MAPK and PI3K/AKT/mTOR cascades[16].

Anti-HER2 drugs targeting the extracellular domain of HER2 have been shown to induce HER2 internalization by downregulating HER2 membrane expression in cells that retain HER2 amplification[14]. This phenomenon was first described in the early 2000s in cell cultures exposed to trastuzumab[2,3], although

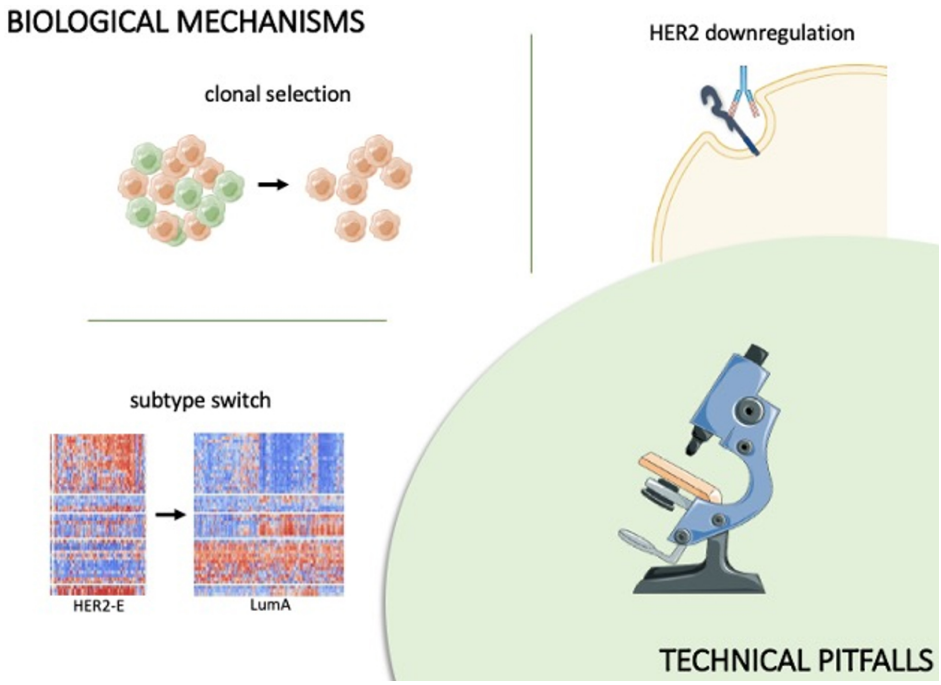

Figure 1. HER2 downregulation, subtype switch, and clonal selection are biological mechanisms potentially responsible for HER2 loss. In parallel, technical pitfalls can cause false-negative results. Figure partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license and adapted from Mathews *et al.*[13] (http://creativecommons.org/licenses/by/4.0/). HER2: Human epidermal growth factor receptor-2.

it has not been confirmed by other studies[14,17]. To date, there is no clear explanation for these contradictory results. Some authors have suggested that immune cells may play a crucial role, since HER2-downregulation seems to occur only when trastuzumab is actively engaged with immune cells[18]. For instance, Shi and colleague observed HER2 downregulation only in co-cultures of cancer cell lines with immune cells and not in culture of cancer cells with trastuzumab only[18]. In particular, they detected an increased level of interferon gamma (IFN-γ) production by immune cells, which in turn activated the signal transducer and activator of transcription 1 (STAT1) that has been assumed to be responsible for HER2 transcriptional downregulation[18,19]. Indeed, the authors found an increased level of STAT1 in cancer cells with HER2 downregulation, whereas inhibition of STAT1 with fludarabine blocked the immune-related HER2 downregulation[18].

More recently, Bon and colleagues reported an even higher HER2 downregulation with the dual blockade by trastuzumab-pertuzumab combination[20]. The authors first observed a reduction in HER2 expression in cell lines after treatment with dual blockade and then demonstrated this *in vivo* by assessing HER2 in four patients with paired biopsies collected before and after exposure to trastuzumab-pertuzumab. HER2 downregulation at the membrane level was observed in all patients, although gene amplification by fluorescence in situ hybridization (FISH) was preserved in two of them[20]. Similarly, HER2-downregulation was observed after treatment with trastuzumab emtansine (T-DM1)[21-23] and has been hypothesized as a potential mechanism of primary or secondary resistance to other anti-HER2 antibody-drug conjugates (ADCs)[22,24]. Of note, the recently presented correlative analysis from the DAISY study, which investigated trastuzumab deruxtecan in patients with HER2-positive, HER2-low (1+ or 2+/FISH-negative) and HER2 0 at IHC, showed a decrease of HER2 expression at progression in 65% of patients[25].

In contrast, TK inhibitors (TKIs), which act at the intracellular level, do not induce HER2 internalization but instead may increase HER2 expression by inhibiting HER2 phosphorylation and preventing receptor ubiquitination and internalization[17]. Indeed, treatment with TKIs might actually result in a marked accumulation of inactive receptors at the cell surface[17]. This phenomenon explains the rationale for combining TKIs with anti-HER2 antibodies, as their antitumoral activity is stronger in combination therapies rather than when single agents are administered[17,26,27].

Intratumor HER2 heterogeneity and clonal selection

Breast cancer is a highly heterogeneous disease, with differences in biology, gene expression profiles, and treatment sensitivity often coexisting in the same tumor area[1]. The exposure to anti-cancer drugs affects these cells differently, killing some cell populations and clonally selecting others.

HER2 heterogeneity can thus result in HER2 loss due to treatment exposure, with HER2-positive subpopulations killed by treatment and resistant HER2-negative cells surviving. Many studies have characterized HER2 heterogeneity in breast cancer using various techniques, but very few of them have examined the correlation with HER2 loss[1,28-30].

In 2009, the ASCO/CAP guidelines defined HER2 heterogeneity as the presence of ≥ 5% to < 50% tumor cells with a ratio ≥ 2.2 when using dual probes or ≥ 6 HER2 signals/cell when using single probes[31]. Two to 4 representative areas of the invasive tumor should be selected and assessed after scanning the entire slide to look for heterogeneity. In 2013, this definition was updated by changing the cut-off value from 5% to 10% and the ratio from 2.2 to 2.0, in line with the updated definition for HER2-positivity[32]. Filho and colleagues applied the first definition to assess HER2 heterogeneity in two different areas of pretreatment biopsies of a cohort of 164 patients enrolled in a single-arm neoadjuvant trial administering T-DM1 plus pertuzumab and its impact on outcomes[28]. Of note, 16 out of 157 evaluable samples were classified as heterogeneous (10%), and none of them achieved a pathologic complete response (pCR) compared to 55% of non-heterogeneous tumors that achieved pCR[28]. Most of the heterogeneous cases were 2+ at IHC (75%) and ER-positive (81%). The authors also assessed heterogeneity as a fraction of the proportion of HER2 amplified cells defined by single-cell FISH and found an even higher correlation with non-pCR. Unfortunately, HER2 status on surgical samples and dynamics of heterogeneity were not reported[28].

Caswell-Jin and co-workers instead applied a whole-exome sequencing approach to evaluate HER2 heterogeneity in pretreatment samples and in different regions of surgical specimens after neoadjuvant anti-HER2 therapy in 5 patients[33]. By comparing the frequency of mutations across spatially distinct regions, they observed clonal replacement in two of five tumors, with mutations detected in surgical specimen that were absent in the pretreatment core biopsy. This suggests selection of resistant cells by neoadjuvant treatment in heterogeneous tumors, although it was not investigated whether this finding correlates with a different receptor profile across heterogeneous areas and HER2 loss was not investigated[33].

Subtype switch

According to gene expression profiles, breast tumors are classified into four major subtypes: luminal A, luminal B, HER2-enriched (HER2-E), and basal type[34-36]. Approximately 50%-60% of HER2-positive breast cancer are classified as HER2-E, with the remaining half distributed among luminal A (15%-20%), luminal B (10%-15%), and basal-like (5%-10%)[37]. HER2-E tumors are characterized by high expression of growth factor receptor-related genes (*ERBB2*, *EGFR*, and/or *FGFR4*) and cell cycle-related genes and low expression of estrogen-related and basal-related genes[38]. These tumor subtypes were initially characterized using microarray analysis evaluating more than 400 genes[35,39], although the PAM50 classifier is currently applied for tumor subtyping in most correlative studies[34].

In a number of studies, platforms for gene expression profiling have been used to assess the tumor intrinsic subtypes of HER2+ BC both before and after neoadjuvant treatment[40-43] [Table 1]. Of note, a subtype switch was observed in a substantial proportion of patients across different drug regimens, with the HER2-E to luminal A switch being the most frequent[40-43]. This finding could be attributed to a decreased expression of genes involved in cell proliferation after treatment exposure. Of note, gene expression analyses cannot distinguish among intra-tumor heterogeneity, stromal alterations, or a true treatment effect, so that stromal contamination might have a role in subtyping after treatment exposure. Interestingly, Brasó-Maristany and colleagues observed that this subtype switch can be reversible after anti-HER2 therapy discontinuation[41].

To our knowledge, none of these correlative studies performed in the context of neoadjuvant clinical trials correlated the subtype switch with HER2 loss on surgery specimens. These data were reported only by Pernas *et al.* in a retrospective study examining the PAM50 subtype in a cohort of 26 HER2-positive BC with paired samples and residual disease (RD) at surgery[44]. In this cohort, most HER2-E tumors (81.8%) converted to non-HER2-enriched, and a conversion to HER2-negative in residual disease was observed in 7/26 patients[44].

TECHNICAL PITFALLS

HER2 status assessment by IHC and ISH represents the standard of care for clinical decision making[8]. Thus far, these are the only two assays that proved to predict response to anti-HER2 therapy in several randomized clinical trials, therefore having solid clinical utility. IHC uses an antibody to assess HER2 protein expression, whereas ISH determines *HER2* copy number using a single probe for copies calculation or a dual probe that includes chromosome 17 centromere probe (CEP17) hybridization to determine the *HER2*/CEP17 ratio[45]. Different categories of ISH-based assays are available, including FISH or bright field-based techniques such as silver (SISH) or chromogenic in situ hybridization (CISH). Although FISH is considered the gold standard and is more used worldwide, SISH and CISH have shown very good concordance with FISH and can be considered as an alternative[8].

Both IHC and ISH have shown a good concordance in assessing HER2 status, but standardization of laboratory testing - including accuracy, reproducibility, and precision - is needed, as technical variabilities can account for both false-negative and -positive results. In particular, ICH is highly dependent on tissue fixation methods, so that variable fixation time and different antibody clones and antigen retrieval methods can lead to incorrect IHC results. Conversely, ISH is less dependent on tissue fixation methods and more reproducible[46,47], although other pitfalls can cause false results such as intratumoral heterogeneity, accidental assessment of in situ rather than invasive lesions, or suboptimal resolution (nonuniform signals, high autofluorescence, poor nuclear resolution, or high background-obscuring signal resolution)[48]. Additionally, ISH assessment on unstained sections stored for prolonged periods can be falsely read as negative. ASCO/CAP guidelines strongly recommend that laboratories performing HER2 testing should participate in regular laboratory inspections and biannual proficiency testing, such as the program offered by CAP[8,32].

The technical variability of these assays accounts for the limited reproducibility of HER2 status across laboratories. Discrepancies in HER2-status between local and central testing have thus been demonstrated in many studies[49-52]. For instance, an analysis of the N9831 intergroup adjuvant trial identified a discordance rate of 18% and 12% for ICH and FISH, respectively, when comparing local and central laboratories, although a high degree of concordance was observed between central and reference laboratories[53]. The meta-analysis by Schrijver *et al.* evaluated instead receptor conversion from primary tumors to distant metastasis and showed a higher rate of discrepancy when HER2 was assessed with IHC

Table 1. Neoadjuvant trials evaluating subtype switch during or after anti-HER2-based therapy

Clinical trial	Patients (*n*)	Treatment	Paired samples (*n*)	Subtype switch
NeoSphere Bianchini *et al.*[42] 2018	417	HD, HPD, HP, PD	166	Significant increase in LumA and decrease in HER2-E and LumB subtypes from baseline to surgery
PAMELA Llombart-Cussac *et al.*[43] 2017	151	HL	57	Baseline: HER2-E 67%, LumA 15%, basal-like 6%, LumB 10% normal-like 2%, D14: normal-like 49%, LumA 25%, HER2-E 18%, basal-like 6%, LumB 3%
CALGB 40601 Carey *et al.*[40] 2016	305	THL, TH, TL	78	Baseline*: HER2-E 15.4% LumA 39.7%, LumB 33.3%, basal-like 5.1%, claudin low 3.8%, normal like 2.6% Residual disease: HER2-E 8.9%, Lum A 48.7%, LumB 5.1%, basal-like 3.8%, claudin low 3.8%, normal-like 2.9%

H: Trastuzumab; P: pertuzumab; D: docetaxel; T: paclitaxel; *considering only patients with residual disease and paired analysis.

(20.8%) instead of FISH (16.3%)[10].

In 2018, the new ASCO/CAP guidelines introduced a 5-group classification for dual-probe ISH results to address specific scenarios of non-univocal interpretation, identifying for all these groups what additional work-up should be performed to obtain the most accurate classification of HER2 status. Groups 1 (HER2/CEP17 ratio ≥ 2, HER2 copy number > 4) and 5 (HER2/CEP17 ratio < 2, HER2 copy number < 4) identify the most frequent and straightforward categories of HER2-positive and -negative samples, respectively, whereas Groups 2-4 include the 5% of cases with a doubtful attribution. These cases frequently stain 2+ by IHC and are referred for a second opinion from a blinded pathologist.

Amplification of *CEP17* represents one of the potential pitfalls causing dual-probe ISH test to fall into these groups. Specifically, cells with co-amplification of *CEP17* and *HER2* are usually characterized by a HER2/CEP17 ratio < 2.0 and an average HER2 signals/cell ≥ 6.0, falling into Group 3. According to the 2018 ASCO/CAP guidelines, these samples should be reported as HER2-positive if IHC 2+/3+ is detected, although it was acknowledged that data on response to anti-HER2 therapy are limited. *CEP17* gains or losses without involvement of the *HER2* gene have also been reported, which lead to an under- or overestimation of HER2 amplification[54]. Of note, multiple gene amplifications on chromosome 17 may potentially involve further telomeric genes alteration (*TOP2*, *RARA*, *GRB7*, and *STARD3*), and this demands careful evaluation of the *HER2* amplicon along with the potentially co-amplified neighboring genes, as well as possibly additional IHC assays for HER2[55].

Further bias in HER2 assessment may result from sampling errors[56]. In the case of metastatic recurrence, guidelines recommend repeating HER2 testing in a metastatic site, if tissue sample is available, although a new biopsy cannot always be feasible[8]. Additionally, false-negative results can be caused by technical pitfalls, as frequently occurs in bone lesions, or by sampling bias due to intratumor heterogeneity[57].

To improve diagnostic precision, some authors recommend also performing ISH in HER2-positive scored as 3+ or 0/1+ at IHC to confirm HER2 status[58].The repeat biopsy, however, is not recommended for receptor re-evaluation, as it does not improve diagnostic accuracy[49].

More recently, HER2 assessment on liquid biopsies has emerged as potential alternative to tissue sampling. Possible sources of HER2 status assessment are circulating tumor cells, cell-free tumor DNA (ctDNA), and extracellular vesicles. The advantage of the method is low invasiveness, albeit copy number assessment is complicated due to the vast background of healthy material, tumor heterogeneity, and high signal-to-noise

ratio. Thus, HER2 assessment on liquid biopsies remains investigational and should not be recommended for clinical decision making[57].

CONCLUSION

Loss of HER2 is not a rare phenomenon, being detected in up to 50% of breast tumors in different settings. Several mechanisms may contribute to this finding, including intratumor heterogeneity, clonal selection, and true biological modifications such as HER2 downregulation or subtype switch. Nevertheless, these associations are mainly speculative, as no study has specifically linked these phenomena to HER2 loss in tissue specimens. On the other side, analytical pitfalls may be responsible for some false-negative results, and some authors have suggested that technical issues are responsible for most cases of HER2 loss. The limited reproducibility of HER2 retesting across laboratories is well-known and can exemplify the technical variability behind these tests[49], especially in immunohistochemical evaluation. In contrast, assessment of gene amplification by ISH is less dependent on technical variables. The ESMO Clinical Practice Guidelines for diagnosis, treatment, and follow-up of primary breast cancer suggest that HER2 gene amplification status may be determined directly from all invasive tumors using any type of in situ hybridization (fluorescent, chromogenic, or silver), completely replacing IHC or only for tumors with an ambiguous (2+) IHC score (II, B)[59].

Many studies investigated receptor conversion both in the neoadjuvant setting and at metastatic relapse[9,11,60,61]. Nevertheless, most of them were small, retrospective, included patients receiving different drug regimens, and applied distinct definitions of HER2-positivity, thus leading to many biases and low statistical power. Additionally, most studies were conducted before the approval of trastuzumab in the (neo)adjuvant settings, so most patients did not receive anti-HER2 therapy[62]. Notably, none of these studies performed correlative analyses to understand the biology behind HER2 loss, but some of them investigated whether analytical problems may have been responsible for some of these cases. When also adding FISH analysis in IHC 0 or 1+ cases, some authors disproved cases of HER2 loss[20,61-64]. This is particularly relevant after administration of trastuzumab or other agents targeting the HER2 extracellular domain, due to the potential downregulation of membrane expression with persisting gene amplification[14,20]. Interestingly, this phenomenon may be reversible, so that HER2 loss may be falsely detected if samples are collected during targeted therapy[41,65]. Although is not possible to distinguish reversible from definitive HER2 loss, it might be argued that patients retaining HER2 amplification at ISH are more prone to restore HER2 expression after stopping anti-HER2 therapy. Unfortunately, timing of HER2 re-overexpression for cases with transient HER2 loss is unknown. To minimize the chance of false-negative results, we believe ISH testing should be performed in all cases of HER2 loss, even in tumors scored as 0 or 1+ at IHC. Indeed, ISH can detect gene amplifications not only in cases of HER2 downregulation but also when technical artifacts lead to a false negative result[49]. Given the efficacy of new anti-HER2 therapies, determination of HER2 addiction/targetability is crucial and may open important therapeutic options with potential impact on survival.

In conclusion, many studies have described HER2 loss in various settings, but correlative studies specifically addressing this phenomenon, i.e., studies collecting and analyzing samples from patients with HER2 loss at different timepoints (before, during, and after therapy), are lacking, and the extent to which technical pitfalls contribute to HER2 loss is unknown.

We believe translational studies that match clinicopathological and biologic features are warranted, to both shed light on this phenomenon and provide guidance on which drugs and strategies may be more effective for patients with HER2 loss.

DECLARATIONS

Authors' contributions

Conceptualization and design: Morganti S
Manuscript writing - original draft: Morganti S, Ivanova M, Ascione L, Vivanet G
Manuscript writing, review and editing: Morganti S, Ivanova M, Ferraro E, Ascione L, Vivanet G, Bonizzi G, Curigliano G, Fusco N, Criscitiello C
Final approval of manuscript: Morganti S, Ivanova M, Ferraro E, Ascione L, Vivanet G, Bonizzi G, Curigliano G, Fusco N, Criscitiello C

Availability of data and materials

Not applicable.

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Conflicts of interest

Curigliano G received honoraria for consulting/advisory role/speaker bureau and/or travel funding from Roche, Lilly, Bristol-Myers Squibb, Pfizer, Novartis, and Seagen; Criscitiello C received honoraria for consulting/advisory role/speaker bureau from Novartis, Eli-Lilly, Pfizer, MSD, Seagen and Roche; Fusco N from Merck Sharp and Dohme (MSD), Boehringer Ingelheim, Novartis, AstraZeneca, and Daiichi Sankyo. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Cancer resistance to immunotherapy: What is the role of cancer stem cells?

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Abstract

Immunotherapy is an emerging form of cancer therapy that is associated with promising outcomes. However, most cancer patients either do not respond to immunotherapy or develop resistance to treatment. The resistance to immunotherapy is poorly understood compared to chemotherapy and radiotherapy. Since immunotherapy targets cells within the tumor microenvironment, understanding the behavior and interactions of different cells within that environment is essential to adequately understand both therapy options and therapy resistance. This review focuses on reviewing and analyzing the special features of cancer stem cells (CSCs), which we believe may contribute to cancer resistance to immunotherapy. The mechanisms are classified into three main categories: mechanisms related to surface markers which are differentially expressed on CSCs and help CSCs escape from immune surveillance and immune cells killing; mechanisms related to CSC-released cytokines which can recruit immune cells and tame hostile immune responses; and mechanisms related to CSC metabolites which modulate the activities of infiltrated immune cells in the tumor microenvironment. This review also discusses progress made in targeting CSCs with immunotherapy and the prospect of developing novel cancer therapies.

Keywords: Immunotherapy, cancer stem cells, immunotherapy resistance, tumor microenvironment

INTRODUCTION

Cancer heterogeneity at the cellular level has recently shifted cancer research from exclusive investigations

of events affecting specific cell types to investigations of events governing all types of cells in the tumor microenvironment[1,2]. Tumor heterogeneity is the concept of morphological and phenotypical variation in cancer cells and, therefore, different proliferation potential. Cancer stem cell theory is a confirmed theory derived from this topic, which characterizes subpopulations of tumor cells that can proliferate extensively[3]. These cells are comparable to normal adult stem cells in that they both undergo self-renewal to maintain the stem cell population. The purpose of this feature is to enable the continuous replacement of cells needed in tissues due to everyday damage, age, *etc*. In cancer stem cells, however, self-renewal has been observed to be the driving force for tumorigenicity and metastasis. Furthermore, there is evidence that cancer stem cell population growth is promoted by oncogenic events induced by chemotherapy, radiation therapy, hypoxia, *etc*., which are generally thought to reduce tumor growth but, on the contrary, increase the risk of cancer recurrence. Cancer stem cells are observed in all cancers[3]. While cancer stem cells were shown to have the ability to differentiate into distinct types of cancer cells within the tumor[4-6], several types of immune cells were shown to control cancer growth in different ways, from directly targeting and killing cancer cells to suppressing the immune attack by modulating other immune cells[7]. While this paradoxical effect of immune cells on cancer growth is critical to developing adequate immunotherapy regimens, the presence of cancer stem cells in the tumor microenvironment is another factor that could affect the response of cancer to immunotherapy and subsequently lead to therapy resistance. This review discusses the distinctive characteristics of cancer stem cells that we believe may contribute to immunotherapy resistance. Along the same lines, we suggest new areas of focus in the field of immunotherapy research, which may help bypass the therapy resistance caused by cancer stem cells.

CANCER STEM CELLS

The presence of stem cells in tumors has shifted laboratory and clinical approaches to cancer research and has given insight into mechanisms contributing to the therapeutic resistance of many cancers[8-12]. Cancer stem cells (CSCs) are the "seed" of the tumor with self-renewal and differentiation properties. They are believed to be present in all cancers. Although there is debate regarding CSC generation, hypotheses include evolving from the de-differentiation of differentiated cells, the division of existent tissue stem cells, and a combination of both rationales[13]. More importantly, CSCs have been observed to resist chemotherapy by at least five main mechanisms: (i) being in a quiescent proliferative state rendering the cells less sensitive to any form of therapy; (ii) activating drug efflux mechanisms; (iii) overexpressing DNA repair mechanisms; (iv) overexpressing anti-apoptotic genes; and (v) releasing cytokines and chemokines rendering other tumor cells resistant to therapy[14]. As the mechanisms by which CSCs affect the development of chemotherapy resistance have been extensively reviewed, we focus on the characteristics of CSCs that may contribute to resistance to immunotherapy and consider recent findings related to cancer immunotherapy and the cancer microenvironment.

THE INTERACTION BETWEEN IMMUNE CELLS AND CANCER STEM CELLS

Current research aims to understand the immune system's role in cancer as it is evident that the immune system does not target cancer cells in the same way that it targets other types of self-altered cells. Cancer can escape from immunosurveillance through a well-described procedure termed "cancer immunoediting", which comprises three main sequential events, namely elimination, equilibrium, and escape, eventually leading to cancer growth[15]. A tumor microenvironment is an ideal place for starting an investigation of factors contributing to therapy resistance because it is a diverse environment in which a variety of cells grow and interact with one another. It has been shown that the tumor microenvironment (TME) is rich with factors that suppress immune cells by inhibiting their growth.

This interaction in TME can involve both innate and adaptive immune systems. Innate immune cells integral for tumor recognition include natural killer (NK) cells, dendritic cells (DC), macrophages, and myeloid-derived suppressor cells (MDSC). Adaptive immune cells include T cells and B cells. The unique properties of CSCs may make them more susceptible or resistant to immune cells. For example, CSCs have been observed to be more susceptible to being killed by NK cells because of their typical presentation of no/low levels of MHC class I[16]. Additionally, CSC-secreted factors can actively modulate the function of immune cells to generate an immune-suppressive environment. Tumor-associated macrophages (TAMs) can be differentiated into an M1 or M2 phenotype via exposure to specific cytokines and metabolites in the TME. M1 macrophages are characterized by phagocytic activity promoting tumor-killing properties, whereas M2 macrophages are characterized by cytokine production promoting an anti-inflammatory response in the TME, thus promoting tumor growth. CSCs have been observed to release many cytokines, chemokines, and soluble proteins to attract macrophages into the TME and polarize them into M2 TAMs[17]. Furthermore, CSC-induced secretion of the immunosuppressive cytokine IL-10 increased the differentiation of $CD4^+CD25^+FoxP3^+$ regulatory T cells[18]. Regulatory T cells are a type of $CD4^+$ T cell that function as an immunosuppressant to prevent inappropriate immune system activation. These immunosuppressive cells can interact with cancer cells in various ways ranging from direct ligand-receptor interactions to systemic regulation[19,20]. While systemic regulation is beyond the scope of our discussion in this review paper, it is essential to mention that altered hematopoiesis in cancer is a growing field of research[21,22] that may contribute to immunotherapy resistance.

CSCs and resistance to immunotherapy

The mechanisms that govern the resistance of cancer to any form of therapy are complicated and numerous. No single mechanism has been shown to fully govern the resistance of a tumor to shrinkage in response to therapy. Instead, a combination of alterations related to surface receptors, secreted factors, and metabolism have been noted in therapy-resistant cells[23]. Therefore, understanding the behavior of all cells in the TME is crucial to fully understanding therapy resistance. As mentioned above, CSCs can contribute to chemotherapy resistance by either modifying their own behavior or by inducing a therapy-resistant phenotype in surrounding cancer cells. It is also important to mention that the mechanisms governing resistance to immunotherapy may differ from those governing resistance to chemotherapy.

Cancer immunotherapy is briefly divided into two main types of interventions: passive and active[24]. Passive immunotherapy strategies aim to compensate for missing or deficient immune functions by directly administering tumor-specific antibodies, recombinant cytokines, or the adoptive transfer of immune cells. Active immunotherapy strategies are designed to stimulate effector functions *in vivo*, e.g., vaccination strategies with tumor peptides[25] or allogeneic whole cells[26], inhibition of immune checkpoints with antibodies, and induction of immune responses with oncolytic viruses. Unlike chemotherapeutic drugs, which directly target cancer cells, the success of immunotherapy relies on the dynamic interaction between cancer and immune cells. Increasing evidence indicates that non-genetic, intrinsic cancer cell alterations play a key role in resistance to immunotherapies and immune evasion. Therefore, understanding therapy resistance in particular requires understanding the interactions among immune cells, cancer cells, and CSCs and the behavior of CSCs in response to immunotherapy, as CSCs are considered the root of cancer.

CSCs were first identified from leukemia cells as a subpopulation of cancer cells that displayed increased tumorigenicity using serial limiting dilution transplantation assays with severe combined immunodeficient (SCID) mice[27]. Thereafter, CSCs in many solid tumors were identified using nonobese diabetic (NOD)/SCID mice[6]. Most of these experiments, using partially immunocompromised mice, demonstrated that a small subset of cancer cells can initiate tumor development. It is still being debated whether the increased tumorigenicity of CSCs attributes to the "stemness" or adaptation ability of CSCs to new

microenvironments[28,29]. Indeed, it has been reported that tumor-initiating cells in human melanoma were not a rare population when transplanted into more highly immunocompromised NOD/SCID IL-2 receptor gamma chain null (NSG) mice[30]. Xenotransplantation of non-CSCs into NSG mice also resulted in tumor formation. However, in tumor tissues developed from non-stem melanoma cells transplanted into NSG mice, the expressions of melanoma markers were no longer retained, suggesting the failure of self-renewal. Moreover, these non-stem cancer cells also failed to pass in NSG mice, whereas CSCs were successively propagated and, more importantly, mirrored the heterogeneity of the parental melanoma[29]. Similar findings have been reported in other models[31]. The current view is that the rarity of cancer cells exhibiting tumor-initiating properties may not be so important for CSC definition[32]. Instead, tumor immune evasion may be a more important characteristic of CSCs. Consistently, tumor tissues exposed to an immune challenge *in vivo* exhibited a CSC-like gene expression profile with increased CSC properties, including tumor initiation ability[33]. Cancer stem cells have characteristics such as slow rates of division, heightened activation of DNA repair mechanisms, cellular plasticity, and microenvironment characteristics including hypoxia and acidosis, which are due to the expression of specific surface markers and secreted and/or enzymatic proteins. Many of these factors may also contribute to the resistance of CSCs to immunotherapy. These mechanisms can be organized into three main categories: (i) mechanisms related to cellular surface proteins; (ii) mechanisms related to released cytokines; and (iii) mechanisms related to metabolic alteration.

Mechanisms related to cellular surface proteins

CSCs are known to express a wide variety of cellular markers that can be used to differentiate them from other tumor cells[34,35]. In addition to "stemness" markers, CSCs also express membrane proteins that play roles in tumorigenesis and/or therapy resistance[36]. This review discusses the markers that are vital in the context of cancer immunology and resistance to immunotherapy.

Immune recognition molecules

One of the mechanisms used by CSCs to evade immune response is the downregulation of major histocompatibility complex class I (MHC I) molecules. This protects them from recognition by $CD8^+$ T cells. For example, enriched cells with stem-related markers isolated from patients with locally advanced head and neck squamous cell carcinoma (HNSCC) showed decreased expression of human leucocyte antigen I (HLA-I) molecule after chemotherapy treatment[37]. Similarly, CSCs isolated from primary melanoma, glioblastoma multiforme (GBM) and lung cancer cell lines displayed lower MHC-I levels than their differentiated counterparts[18,38-40]. Downregulating MHC-I may make CSCs more susceptible to being killed by NK cells. However, CSCs from acute myeloid leukemia (AML)[41], GBM[42], and breast cancer (BC)[43] were found to escape NK cell-mediated killing by the downregulation of activating NKG2D ligands. Lastly, the upregulation of the "don't eat me" signal, CD47, is another way for CSCs to evade immune control[44]. The blocking of CD47 was shown to enable macrophage-mediated phagocytosis of CSCs from pancreatic ductal adenocarcinoma (PDAC), AML, and hepatocellular carcinoma (HCC), and thus it promotes their elimination[45-47].

Immune checkpoint proteins

PD-L1/PD-1 has been well established as an immune checkpoint to control T cell function. CSCs isolated from GBM, BC, CRC, and HNSCC can evade immune surveillance by increasing the expression of the immune checkpoint ligand PD-L1[48], which binds to its receptor, PD-1, expressed on T-cell surfaces, thus inducing their exhaustion[48]. Another immune "brake" is represented by T cell immunoglobulin mucin-3 (TIM-3), a specific surface molecule found on leukemic stem cells[49]. This receptor was described as responsible for T-cell suppression and MDSC expansion. Similarly, it was observed that B7-H4 promotes brain CSC tumorigenicity while negatively regulating T cell-mediated immunity[50].

Immuno-stimulatory molecules

CD80 is a co-stimulatory molecule known for its role in T-cell activation. It is primarily expressed by immune cells but has been shown to be expressed in cancer cells including melanoma, colorectal cancer, and some tumor-initiating stem cells[51,52]. CD80 is an important cellular surface protein that has been shown to contribute to stem cell resistance to immunotherapy[52]. In one study conducted on a model of epidermal squamous cell carcinoma, CD80 in CSCs was shown to be indispensable for CSC resistance to adoptive cytotoxic T cell transfer therapy (ACT)[52]. Interestingly, CD80 was also observed to interact with cytotoxic T lymphocyte antigen-4 (CTLA-4), which is known to suppress T cell function[53]. Upon engaging CTLA-4, CD80-expressing CSCs directly dampen cytotoxic T-cell activity, which provides evidence for the importance of CD80 in immunotherapy resistance. The mechanism of action, however, needs to be confirmed by future research. In the same study, the authors selected CSCs that displayed sensitivity to TGFβ and studied the resistance of those CSCs to adoptive cell transfer (ACT) therapy. TGFβ responding CSCs were shown to be resistant to ACT, and, interestingly, CD80 expression was observed to be indispensable for resistance. However, interactions between these two proteins have not been studied[52]. Future research is required to understand the possible overlap in the mechanisms governing TGFβ sensitivity and CD80 in these resistant cells. Moreover, the clinical feasibility of specifically targeting CSCs' CD80 while sparing CD80 expressed by other immune cells is also not well understood. In a more recent study, both *in vitro* and *in vivo* deactivation of CD80 were associated with higher immunogenicity manifested by the activation of immune cells[54]. This immunogenicity was antagonized by blocking CTLA-4[54]. Although the aforementioned study does not specifically target CSCs, it confirms two important concepts governing the mode of action of CD80 (shown in the previously mentioned study to be highly expressed by TGFβ sensitive CSCs): (i) CD80 has an anti-immunogenic role in at least some types of cancer; and (ii) CD80 exerts its anti-immunogenic functions, at least in part, through CTLA-4.

Apart from the above-mentioned mechanisms, CSCs further interact with immune cells in order to create a suitable microenvironment for its survival and self-renewal. Studies have shown the importance of TAMs in maintaining tumor homeostasis and immune suppression. One such interaction involves the myeloid cell marker CD11b on TAMs and CD90 on cancer stem cells, accompanied by EphA4 and epinephrine leading to the release of several other immunosuppressive cytokines that help maintain cancer stemness and survival[55]. CSCs can also interact with $CD66^+$ neutrophils to prevent their maturation into N2 neutrophils alongside blocking T cell IFNγ-mediated killing of cancer cells[56,57]. Another study showed the importance of CSCs surface marker CD133, which prevents DC maturation and thus downregulates T cell-mediated response[58].

Mechanisms related to released cytokines

The release of cytokines has been observed to have paradoxical effects on immunotherapy resistance. While proinflammatory cytokines are required for adequate function of T cells in ACT and in CTLA-4/PD-1 targeting therapies, they can induce stemness of CSCs, promoting therapy resistance[36]. Many cytokines have dual roles in promotion of CSC self-renewal and immune evasion and immune suppression. In this context, two main therapy options arise: (i) moderating the proinflammatory environment in a way that can induce/maintain stemness; and (ii) antagonizing the proinflammatory environment in a way that can block stemness, at least partially, yet create an immunosuppressive environment that may not be favorable for immunotherapy.

Tumor microenvironments in solid tumors are rich in many cytokines, including IL-1, IL-6, IL-4, IL-8, granulocyte-CSF, MF inhibitory cytokine-1 (MIC-1), and TGF-β, which can impair the anti-tumor immune responses and shield them from T-cell infiltration[59]. IL-1β is responsible for the infiltration of myeloid cells, primarily MDSCs, $CD11b^+Gr\text{-}1^+$ granulocytes, and $CD11b^+F4/80^+Gr\text{-}1^{-/low}$ TAMs in the tumor

microenvironment, thereby promoting tumor growth, progression, and poor prognosis[60,61]. IL-6 produced in the tumor microenvironment by CSCs has been shown to enhance the neoplastic progression of tumors. STAT3 activation by IL-6 in TAMs and MDSCs induces the expression of VEGF and bFGF in a positive feedback mechanism promoting angiogenesis[62]. Apart from regulating the TAMs and MDSCs, IL-6 has also been shown to interfere with the antigen presentation of DCs that are primarily important for the activation and priming of $CD8^+$ T cells (cytotoxic T cells)[62]. Furthermore, IL-6 signaling also induces the polarization of immature myeloid cells to macrophages or MDSCs instead of antigen-presenting DCs, which in turn contributes to tumor progression. IL-8, for example, has been shown to recruit and activate the differentiation of immature neutrophils into pro-tumor neutrophils in the tumor microenvironment, thereby helping tumor progression and angiogenesis[63]. These pro-tumor neutrophils can suppress the adaptive immune response by secreting arginase 1, which cleaves the arginase required by the cytotoxic T cells to function[64,65]. CSCs were shown to promote the polarization of macrophages toward an M2 phenotype by the production of TGF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage colony-stimulating factor (M-CSF)[66] and via the cyclooxygenase (COX)-2/PGE2 pathway[67]. In turn, M2 TAMs support the expansion and drug resistance of CSCs by producing the cytokine IL-6[68]. Administration of TGF-β inhibitors eliminates TGF-mediated immunosuppressive effects on the immune system, thereby enhancing immunotherapy efficiency[69].

Apart from their role in immune suppression, many cytokines, including TNFα[70], IL-17[71], IL-1[72], and IL-8[64], are known to activate CSC formation and renewal. For example, a recent study showed that IL-17 could activate the autophagy of CSCs through one of its receptors, IL-17RB, in a gastric cancer model[73,74]. While autophagy is a cell death mechanism, it may be indispensable for the self-renewal of CSCs and can be considered as a homeostatic mechanism through which the tumor's pool of CSC is replenished.

Mechanisms related to metabolic alteration

Although poorly studied, metabolic alterations in CSCs might be vital for the adequate understanding of many aspects of the tumor microenvironment[75,76]. Metabolic alterations in CSCs have been shown to govern cancer-related mechanisms that may serve purposes beyond energy production[76]. In other words, metabolic alterations might go hand in hand with alterations in both stemness and growth potential. For example, the overproduction of lactate has always been thought of as a demonstration of the tumor's adaptation to hypoxia and is now known to have roles in the self-renewal of CSCs. We focus our discussion on CSC metabolic plasticity, which may govern resistance to immunotherapy and be a potential therapy target[77].

Excessive lactate production

Although studies have shown that CSCs can undergo oxidative phosphorylation, the glycolysis/lactate production rate has been shown to be significantly higher in CSCs than in non-CSCs of a tumor[78]. Moreover, CSCs were also shown to be activated by lactate[79]. One recent study showed that lactate, produced by differentiated cancer cells from a colorectal cancer patient, was shown to promote the self-renewal of CSCs in organoids[80]. Therefore, lactate represents an important metabolite that is secreted by CSCs and responded to by CSCs. Lactate can induce the accumulation of MDSCs through granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6 and enhance the immunosuppressive phenotype of MDSC through the G-protein-coupled receptor 81 (GPR81)/mTOR/HIF-1a/STAT3 pathway[81,82]. In addition, lactate-induced HIF-1a activates the CCL20/CCR6 axis by inducing myeloid trigger receptor-1 (TREM-1) expression in TAMs, attracting aggregation and initiating immunosuppressive effects of Treg. A recent study showed that lactate-derived lactylation of histone lysine residues serves as a new epigenetic modification that directly stimulates gene transcription from chromatin[83]. Histone lysine lactylation has been shown to promote M2 activation, but the exact mechanism is not well understood[83]. Besides, lactate

inhibits the expression of activated receptor NKp46 in NK cells[81]. What is yet to be determined, however, is whether targeting lactate metabolism would affect the response of tumor cells to immunotherapy by antagonizing the stemness in CSCs.

Lactic acid accumulation in the tumor microenvironment is a well-established immunosuppressive strategy employed by CSCs. Studies have shown that the increased accumulation and production of lactate can hinder tumor infiltration of NK cells and T cells[84] as well as inhibit proliferation and the production of cytokines by T cells[85]. A very recent article also discusses the role of lactate in the tumor microenvironment regulating T-cell redox functions and suppressing T-cell proliferation, wherein the authors showed that inhibiting lactate production by cancer cells can improve T-cell functions in adaptive T-cell therapy and overcome immunotherapeutic resistance[86]. The acidic microenvironment also impairs IL-2 and IFNγ production by T cells, thus reducing the cytotoxic effects of effector T cells[87].

Retinoic acid metabolism in CSCs

ALDH1 is a putative CSC marker in a variety of solid cancers including pancreatic cancer, ovarian cancer, uterine cancer, and breast cancer[88]. Several isoforms (ALDH1A1, ALDH1A2, and ALDH1A3) play a role in retinoic acid (RA) formation through oxidation of all-trans-retinal and 9-cis-retinal that are involved in retinoid signaling, which has been related to the stemness of CSCs. Although it has been observed that RA has anti-tumor and anti-CSC capabilities, there is evidence that its role is manipulated by TME and associated with the ALDH1A3 receptor to promote CSC stemness[89]. Sullivan *et al.* observed that RA metabolism via the ALDH1A3 receptor transcriptionally upregulated tissue transglutaminase in mesenchymal glioma CSCs, an enzyme associated with aggressive tumors and upregulation of $CD44^+$ glioma CSCs[90]. They further noted that glioma CSCs regulated this mechanism by promoting the transcription of RA-inducible genes. Marcato *et al.* found a similar correlation regarding the promotion of RA-induced genes in aggressive and/or triple-negative breast cancer cell lines upon ALDH1A3 and RA interaction[91].

This interaction may be a potential factor in immunotherapeutic resistance. Terzuoli *et al.* found that ALDH3A1 overexpression in both melanoma and non-small-cell lung carcinoma transcriptionally modified the cells to CSC phenotype with upregulation of PDL-1 and EMT markers and an increase in proinflammatory immunosuppressive factors including NFκB, prostaglandin E2, IL-6, IL-13, and COX-2[92]. Additionally, Devalaraja *et al.* uncovered that TME-induced tumor-derived RA induced by IL-13 in sarcoma cells supported immunosuppression via inhibiting monocyte differentiation of antigen-presenting dendritic cells (DCs) and, instead, promoted differentiation of M2 TAMs[93]. It is likely that CSCs contribute to this interaction as studies have observed their involvement in both inhibiting DC maturation and promoting M2 TAM differentiation from monocytes[94].

Altered metabolic pathways in TAM

Solid tumors are known to have special metabolic characteristics that would allow them to face challenges related to hypoxia and/or shortage of nutrients[77,95]. It has been shown that hypoxia in the tumor microenvironment preferentially upregulates OXPHOS and FAO metabolic pathways in the M2 TAMs. This causes the accumulation of metabolic byproducts such as glutamine, a-ketoglutarate, and succinate, resulting in activation of the HIF-1α-mediated cell-self-renewal signaling pathway. Furthermore, tumor-associated macrophages undergo "M2 polarization" by secreting IL-6 to promote PI3K/AKT phosphorylation. This promotes protein kinase 1 (PDK1)-mediated phosphoglycerate kinase 1 (PGK1)-catalyzed glycolysis of macrophages[96]. Interestingly, IL-6, as discussed in a previous section, can enhance the stemness of CSCs and CSC-dependent chemoresistance through STAT3 transcription factor activation. Other studies have shown a mutually symbiotic relationship between CSCs and M2 TAMs, in that CSCs

were considered to play an active role in M2 polarization, resulting in inhibition of antigen presentation and anti-tumor cytotoxic $CD8^+$ T-cell responses[17]

Immunotherapeutic strategies to target CSCs

To achieve complete regression of tumors, CSCs have to be targeted for therapy. Compared to differentiated counterparts, CSCs are known to express a different set of genes that can potentially serve as tumor antigens. Although all over-expressed antigens might not be strong immunotherapeutic targets, certain other types of overexpressed antigens have been studied, such as ALDH1A1 and hTERT in $CD44^+$ breast cancer CSCs[97], HER2 proto-oncogene in glioma CSCs[98], and CEP55 and COA-1[99] in colon CSCs. These overexpressed antigens could be novel targets for the development of CSC-associated immunotherapeutic strategies. Dendritic cells (DCs) are antigen-presenting cells in the immune system that induce primary immune system responses. In the creation of DC-based vaccines, DCs are primed with tumor antigens, such as fusion with tumor lysates and transfection of certain peptide sequences to induce T-cell activation upon vaccination[100]. Examples of recent vaccines being studied targeting CSCs include DCs primed with CSC lysate, Panc-1 CSC lysate, NANOG peptide, and $ALDH^{high}$ CSCs[101]. In a study conducted by Yin *et al.*, DCs were primed with pancreatic CSC lysates identified via culturing Panc-1 cells with a non-adherent sphere culture system due to the heterogeneity of surface markers. Upon co-culturing with lymphocytes, proliferation and significant activation of lymphocytes were observed along with the secretion of tumor-killing cytokines INF-γ and IL-2. Significant cytotoxic effects induced by the primed DCs were also observed[102].

Oncolytic virotherapy utilizes viruses engineered to replicate within tumor cells via the cytolytic pathway. The virus is selected regarding the type of cancer and its known receptors to increase infectivity of the tumor cells[103]. Examples of viruses used include the herpes simplex virus, adenovirus, vaccinia virus, and measles virus[101]. Sato-Dahlman *et al.* conducted a study designing and testing a form of oncolytic virotherapy for colorectal cancer with an adenovirus. The adenovirus was selected in particular because of its capability for efficient transduction in cells with its receptor to combine with the engineered specificity for the CD133 receptor known to be upregulated in colorectal cancer, among other types of cancer. The transmembrane receptor CD133 has been highly studied as a cancer stem cell marker, and its upregulation has been associated with the maintenance of self-renewal and metastasis. The results support increased infectivity and lysis of $CD133^+$ cells *in vitro* and *in vivo*[104].

Some CSC-specific surface markers can be used as specific targets for chimeric antigen receptor T cell (CAR-T) therapy to eliminate CSCs. In addition, the expression of MHC molecules on the surface of CSCs is low, which causes MHC restriction when immunotherapy is used to target CSCs[39]. However, in CAR-T therapy, CAR-T cells can recognize the target antigen with no MHC restrictions, which endows some advantages for the application of CAR-T therapy to eliminate CSCs. Recently, the clinical application of CAR-T therapy has made an unprecedented breakthrough in the treatment of hematological diseases[105]. The safety and feasibility of CAR-T therapy in the treatment of solid tumors have also been confirmed[106]. The discovery of surface markers of CSCs provides specific therapeutic targets for the treatment of CSCs. Many previous experiments have identified the expression of CD133, CD90, ALDH, and EpCAM in CSCs of many types of cancer[107-109]. These markers can be used as an important molecular target for CAR-T cells to kill CSCs in order to achieve the therapeutic effect of eliminating CSCs and inhibiting tumor recurrence and metastasis. In addition, certain molecular markers expressed in common tumor cells, such as epidermal growth factor receptor variant III (EGFRvIII), chondroitin sulphate proteoglycan 4 (CSPG4), human epidermal growth factor receptor 2(HER2), NKG2D ligands (NKG2DLs), *etc.*, are also expressed on the surface of tumor stem cells[110]. The construction of CAR-T cells with molecular markers of CSCs as targets has a certain theoretical effect on the elimination of CSCs.

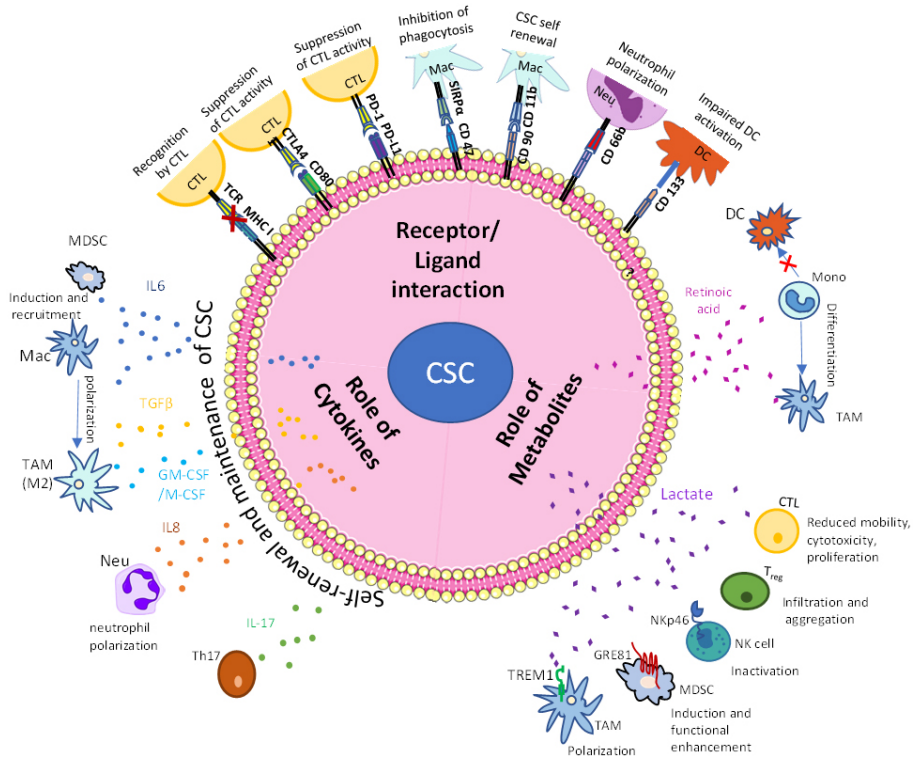

Figure 1. CSC-mediated immune escape and self-renewal. The tumor microenvironment is home to a lot of immune cells, which contribute to cancer cell survival and proliferation. The CSCs can self-renew themselves due to their stem-like properties and are also aided by different cytokines in the TME. Metabolic products from the CSCs, such as retinoic acid and lactate, are also involved in their self-renewal. Other immune cells in the TME, such as MDSCs, immature neutrophils, and TAMs, are also involved in tumor proliferation by dampening the cytotoxic effects of killer T cells and promoting regulatory T cells. CSCs: Cancer stem cells; TME: tumor microenvironment; TAMs: tumor-associated macrophages; CTL: cytotoxic T lymphocyte; Mac: macrophage; Mono: monocyte; Neu: neutrophil; DC: dendritic cells; Treg: regulatory T cell.

TIL follows the same methodology, but it isolates T cells with tumor-specific epitopes to target antigens rather than the CAR receptor. Chen *et al.* employed this strategy in their observation of the synergistic effect between γδ T cells and $CD8^+$ T cells in killing CSCs. This was achieved because γδ T cells secrete interferon gamma (IFN-γ), resulting in the upregulation of MHC class I and CD54/ICAM-1 in CSCs and, thereby, allowing CSCs to become more susceptible to $CD8^+$ T cell-mediated cytotoxicity[111].

While low expression of MHC-I may limit CD8 T-cell recognition and response to CSCs, the lack of MHC-I molecules should, in turn, promote NK cell activation, representing an alternate immunotherapeutic target[112]. In regard to adoptive immunotherapy of NK cells, NK cells have been identified to contain activating and cytotoxic receptors that can bind with tumor-specific stress-induced ligands, thereby increasing antigen recognition[113]. There is evidence that NK cells may preferentially target CSCs because of both this and their ability to identify the lower expression of MHC class I molecules that provide CSCs their non-proliferative properties. This was supported in the study conducted by Ames *et al.* in their identification of CSC markers $CD24^+/CD44^+$, $CD133^+$, and aldehyde dehydrogenase bright present on NK cells *in vitro* and *ex vivo*[114]. CSC ligands MICA/B, Fas, and DR5, involved in NK activation, were also noted to be upregulated. The mechanism for this observation was further investigated by studying the NKG2D pathway that is important for the binding of MHC class proteins during cellular stress for increased cytotoxicity. Significant *in vivo* studies have found reduced CSC population and tumor size in NSG mice with human pancreatic tumors[114].

CONCLUSION AND FUTURE PERSPECTIVES

Many studies performed in the past decade confirm the importance of the whole microenvironment in driving cancer growth and resistance to therapy. As immunotherapy targets a very fundamental aspect of the tumor microenvironment, namely the interaction between immune cells and cancer cells, investigating the tumor microenvironment is critical for an adequate understanding of therapy options and therapy resistance. This review focuses on CSC characteristics that we think might govern the resistance of cancer to immunotherapy [Figure 1]. While the focus is on the expression of cellular markers/release and response to cytokines/metabolic reprogramming, other mechanisms might govern CSCs' ability to bypass or help cancer cells bypass immunotherapy. In addition to answering the questions raised in this review paper, future research needs to focus on other aspects that might govern resistance. These include the systemic control of the hematopoiesis characteristic of cancer and the common markers between CSCs and embryonic stem cells, which might allow for escaping immune surveillance and, therefore, escaping immunotherapy.

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Authors' contributions

Drafted the manuscript, read and approved the final manuscript: Gupta G, Merhej G, Saravanan S, Chen H

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

Open Access

Association between genome-wide epigenetic and genetic alterations in breast cancer tissue and response to HER2-targeted therapies in HER2-positive breast cancer patients: new findings and a systematic review

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Abstract

Recent evidence suggests that genetic and epigenetic mechanisms might be associated with acquired resistance to cancer therapies. The aim of this study was to assess the association of genome-wide genetic and epigenetic alterations with the response to anti-HER2 agents in HER2-positive breast cancer patients. PubMed was screened for articles published until March 2021 on observational studies investigating the association of genome-wide genetic and epigenetic alterations, measured in breast cancer tissues or blood, with the response to targeted treatment in HER2-positive breast cancer patients. Sixteen studies were included in the review along with ours, in which we compared the genome-wide DNA methylation pattern in breast tumor tissues of patients who acquired

resistance to treatment (case group, *n* = 6) to that of patients who did not develop resistance (control group, *n* = 6). Among genes identified as differentially methylated between the breast cancer tissue of cases and controls, one of them, *PRKACA*, was also reported as differentially expressed in two studies included in the review. Although included studies were heterogeneous in terms of methodology and study population, our review suggests that genes of the PI3K pathway may play an important role in developing resistance to anti-HER2 agents in breast cancer patients. Genome-wide genetic and epigenetic alterations measured in breast cancer tissue or blood might be promising markers of resistance to anti-HER2 agents in HER2-positive breast cancer patients. Further studies are needed to confirm these data.

Keywords: Breast neoplasms, epigenetics, genetics, HER2 inhibitors, treatment response, biomarkers

INTRODUCTION

The human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor and belongs to the epidermal growth factor receptor (EGFR) family[1]. It comprises an extracellular domain (ECD), a transmembrane segment, and an intracellular region[2]. *HER2* gene amplification and receptor overexpression, which occur in approximately 15%-20% of breast cancer patients, are important markers for poor prognosis, including a more aggressive disease and shorter survival[3]. In addition, HER2-positive status is considered a predictive marker of response to HER2-targeted drugs[4]. Detection of receptor overexpression via immunohistochemistry (IHC) and/or *HER2* gene amplification using *in situ* hybridization (ISH) techniques in breast cancer tissue, including fluorescent ISH (FISH), determines patients' eligibility to receive anti-HER2 therapies[5]. Food and Drug Administration (FDA)-approved anti-HER2 agents currently used in clinical settings in combination with chemotherapy comprises recombinant monoclonal antibodies that bind the ECD of HER2, such as trastuzumab (Herceptin®), pertuzumab (Perjeta®) and trastuzumab emtansine (T-DM1, Kadcyla®), and small molecule tyrosine kinase inhibitors, like lapatinib (TYKERB®), that inhibit enzyme function of the intracellular catalytic domain of HER2 and other EGFR members[6,7].

Although targeted treatment with anti-HER2 agents has significantly improved the disease-free and overall survival rates of metastatic and early-stage HER2-positive breast cancer patients[8-11], resistance to anti-HER2 therapy, both primary and acquired, has emerged as a major clinical problem in the treatment of HER2-positive breast cancer patients[12-14]. Even though several molecular mechanisms of resistance to anti-HER2 agents have been proposed in preclinical models, no clinically applicable strategy to overcome resistance to these targeted treatments has been identified yet[15]. Therefore, there is an urgent need to identify reliable predictive molecular markers of treatment failure with the ultimate goal of developing targeted drugs that can overcome resistance.

Studies indicate that genetic factors, including single nucleotide polymorphisms (SNPs), copy number variations (CNVs), *HER2* mutations, and HER2 splice variants, might influence treatment effectiveness toward targeted therapies in HER2-positive breast cancer patients or HER2-positive breast cancer cell lines[16-28]. Recent evidence suggests that epigenetic regulatory mechanisms, including DNA methylation and microRNAs (miRNAs), might play a role in acquiring resistance to cancer therapies[29-32]. DNA methylation occurs through the covalent attachment of a methyl group on cytosine residues in CpG dinucleotides and contributes to transcriptional regulations[33]. While DNA methylation in the immediate vicinity of the transcriptional start site (TSS) generally represses gene expression, methylation in the gene body (far from annotated TSS) may stimulate elongation and is, therefore, positively associated with gene expression[34,35]. miRNA are approximately 22 nucleotides long non-coding RNAs that regulate gene expression in a sequence-specific manner[36].

The aim of this pilot study was to analyze the association between DNA methylation patterns in breast cancer specimens and response to trastuzumab in a cohort of 12 trastuzumab-treated, non-metastatic HER2-positive breast cancer patients. Additionally, a systematic review that combines these findings with all available published results on the association of genome-wide genetic and epigenetic alterations in breast cancer tissue or blood with response to anti-HER2 treatment in HER2-positive breast cancer patients is also reported.

MATERIAL AND METHODS

Pilot study of new findings

Study population and data collection

The study population consisted of 12 women (six cases and six controls) selected among 106 trastuzumab-treated patients with non-metastatic, HER2-positive breast cancer diagnosed between July 1, 2005 and December 31, 2010 at the Centre des Maladies du Sein, a specialized breast center in Quebec City, Canada. Information on tumor characteristics and prognostic factors at the time of diagnosis (baseline) and follow-up information were collected from medical records. The clinical endpoint in this study was disease-free survival (DFS). All breast cancer recurrences (locoregional, contralateral breast, and distant) were considered as events, whereas death (from any cause) before recurrence and loss to follow-up were considered as censoring events.

Over a mean follow-up period of 6.22 years, 22 patients out of 106 experienced recurrence. Eight cases were randomly selected among all patients who developed recurrence during follow-up and six had a sufficient amount of primary breast cancer tissue available for DNA extraction (see below). Of note, baseline characteristics of the six selected cases were comparable to the total population of cases (n = 22) for all characteristics except tumor grade (the proportion of grade III tumors among the selected cases was 67% *vs.* 41% for the total population of cases) [Supplementary Table 1]. For each case, one control was selected from the 84 patients who had not developed recurrence and were alive at the date of the case's recurrence. Controls were matched to cases for the following factors: age at diagnosis (with 5-year age categories), estrogen receptor (ER) status, year of diagnosis (with 2-year categories), and menopausal status. The number of samples used was determined upon the availability of samples and not evaluated using a statistical sample size calculation. All patients provided written informed consent. Ethical approval of the study was obtained from the Research Ethics Committee of the Centre de Recherche du CHU de Québec (# 2016-2802).

Gene methylation assessment

To ensure that DNA methylation was analyzed to the greatest possible extent in breast cancer tissue and to reduce contamination with other cell types (lymphocytes, adipocytes, fibroblasts), tissue microarray (TMA) blocks containing formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue cores (1 mm in diameter) were constructed for each patient, as previously described[37]. From each TMA block, one section was stained with hematoxylin and eosin (H&E) to verify the cellular composition of the cores. Cores were removed from TMA blocks if they contained abnormal tissue or if epithelial tumor tissue occupied < 70% of the core area before proceeding to DNA extraction. H&E sections were prepared from different levels of the TMA blocks: at the beginning, at regular intervals (every tenth 10-µm-thick serial section), and after the last section. DNA was extracted from tissue cores using GeneJET FFPE DNA Purification kit (ThermoScientific, Ottawa, Canada) with minor modifications to the manufacturer's instructions in which samples were incubated with Digestion buffer for six minutes and incubated with Proteinase K solution for 180 minutes.

DNA samples were sent to Génome Québec Innovation Center (Montreal, Canada). Methylation was measured with the Illumina HumanMethylation450 BeadChip array (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions for the bisulfite treatment, Infinium FFPE quality control (Illumina FFPE QC kit, Illumina, Inc., CA, USA), and DNA restoration. This BeadChip interrogates 482,421 CpG sites, 3091 non-CpG sites, 65 random SNPs, and covers 21,231 RefSeq genes. It uses two distinct oligonucleotide probes (Infinium I and Infinium II) to assess methylation levels[38].

Statistical analysis

Raw β-values, defined as the ratio of the methylated probe intensity to the overall intensity (sum of the methylated and unmethylated probe intensities)[39], were imported into the R statistical programming environment (version 3.2.2). Since M-values [logit transformed β-values, calculated as $M=\log_2(\frac{\beta}{1-\beta})$] are considered more reliable in the detection rate and true positive rate for both highly methylated and unmethylated CpG sites compared to β-values[39], M-values were used for statistical analyses.

Quality control was performed with the qcReport function from the minfi package, and none of the 12 samples were excluded due to bad quality control. The Dasen method from the WateRmelon package, also known as data-driven separate normalization, was used to background correct and quantile normalize data based on methylated and unmethylated intensities, separately, by probe types (Infinium I and II)[40]. A probe filtration step was performed to remove CpG sites corresponding to probes that could affect our analysis, including probes with bad detection (detection *P*-value > 0.01); unique probes having a common single nucleotide polymorphism (SNP) in European individuals at the interrogated CpG loci or the single-base extension according to the list published by Chen *et al.*[41]; probes that can hybridize to multiple loci also listed by Chen *et al.*[41]; and probes located on X and Y chromosomes. A total of 76,161 unique probes were removed, leaving 406,260 autosomal probes for the analysis. Data were verified for confounding batch effects due to separate chips[42], and none were observed. All samples passed quality-control tests and were therefore retained in the analysis.

Baseline characteristics between cases and controls were compared using Fisher's exact test for categorical variables, Student's *t*-test for follow-up time and Wilcoxon-Mann-Whitney test for the other continuous variables. The difference in global methylation levels between median M-values of cases and controls was assessed using a Wilcoxon signed-rank test for paired samples. Differentially methylated probes (DMPs) were identified using LIMMA (robust linear regression method), taking into account the matching factors between cases and controls (i.e., age at diagnosis, ER status, year of diagnosis, and menopausal status). Multiple testing correction was performed using false discovery rate (FDR) estimation (cut-off < 5%). In addition, we used a $\log_2$-fold change |log2FC| (i.e., the difference between mean M-values measured in breast cancer tissues of resistant patients and controls) > 2.0 as a cut-off to identify probes that were strongly differentially methylated between cases and controls.

Systematic review of published findings

A systematic review was conducted and reported according to the 2020 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines[43].

Eligibility criteria

Population: We included studies of HER2-positive breast cancer patients treated with any type of anti-HER2 agent (trastuzumab [Herceptin®], lapatinib [TYKERB®], pertuzumab [Perjeta®], trastuzumab emtansine [trastuzumab-DM1, Kadcyla®], erlotinib [Traceva®], gefitinib [Iressa®]) regardless of age, stage, and menopausal status.

Exposure: To be included, a study had to measure response-specific survival using including pathologic complete response (pCR), disease-free survival (DFS), progression-free survival (PFS), or event-free survival (EFS).

Outcome: We considered all assessments of genetic and epigenetic alterations at the genome-wide level in breast cancer tissue or blood, whatever the measurement method.

Types of studies: Any observational or randomized controlled study that assessed the association between genome-wide genetic and epigenetic alterations in breast cancer specimens or blood and the response to anti-HER2 agents in HER2-positive breast cancer patients was eligible. Case reports were excluded.

Only full available articles in English were included.

Information sources

Dragic D searched the PubMed biomedical database from inception to the search date of March 25, 2021, to identify eligible studies.

Search strategy

The search strategy was developed by Dragic D and Furrer D, approved by Diorio C, using controlled vocabulary search terms and free-text words related to HER2-positive breast cancer, genome-wide genetic and epigenetic alterations and treatment outcome [Supplementary Table 2]. No restrictions regarding language were applied.

Selection process

The references identified by the search strategy were selected according to the predefined eligibility criteria in a two-step process: titles and abstracts were screened by one author (Dragic D), and full texts of retained articles were examined by two authors (Dragic D and Furrer D). Disagreements between the two authors were discussed until a consensus was reached, and whenever required, a third review author (Diorio C) was consulted.

Data collection process

We designed a data extraction form for this review. Data were extracted by two authors (Furrer D and Dragic D) for half of the included studies. A third author (Diorio C) was consulted when discrepancies between both authors could not be resolved. For the remaining studies, the extraction was done by one author (Dragic D), and when needed, a second author (Diorio C) was involved. Additionally, the authors of studies of interest that lacked data to evaluate eligibility (n = 1) or other measures needed for this review (n = 3) were contacted to obtain the necessary information.

Data items

For all selected articles, study characteristics (study design and sample size), patient's characteristics (age, stage, ER and PR status, menopausal status, and treatment received), assessment of genetic and epigenetic alterations (tissue processing, DNA, RNA or miRNA extraction method, assessment method, and parameters used), as well as statistical methods and study results, were collected. The study's definition of response to targeted treatment was recorded.

Risk of bias assessment

Studies included in the review were assessed for risk of bias using the "Risk Of Bias in Non-randomized Studies of Interventions (ROBINS-I) tool[44]. The following domains were assessed: selection of participants in the study, exposure measurement, outcome measurement, potential confounding factors, missing data, and selective reporting.

Assessment of the risk of bias was done by two authors (Furrer D and Dragic D) for half of the included studies. Inconsistencies were discussed to reach a consensus. For the remaining studies, the assessment was done by one author (Dragic D), and when required, a second reviewer (Furrer D) was consulted.

Assessment of heterogeneity

Differences between studies, including study design, patient characteristics (age, menopausal status, ethnicity, and treatment received), tumor characteristics (stage, ER status), assessment of genome-wide genetic and epigenetic alterations (tissue processing, extraction method, and measurement method), and different levels of risk of bias were considered for exploring possible sources of heterogeneity.

Synthesis methods

Considering that high heterogeneity between studies was expected, quantitative data synthesis was not considered appropriate. Instead, we adopted a qualitative systematic review approach to investigate the relationship between epigenetic and genetic alterations and response to HER2-targeted therapies in HER2-positive breast cancer patients. The selection process was detailed using the PRISMA 2020 flow diagram. Extracted data were first reported in a table gathering summary characteristics of all included studies. Additional information specific to the epigenetic or genetic method used was detailed in several tables. Results and genes identified by several studies were also highlighted in a table. If pathway analysis was not presented, we performed pathway analysis using the list of the differentially expressed genes reported by the study authors and the PANTHER online software (Protein Analysis Through Evolutionary Relationships). P-values < 0.05 were considered significant.

RESULTS

Pilot study of new findings

Genome-wide DNA methylation data in 12 breast cancer specimens were obtained from six trastuzumab-treated HER2-positive breast cancer patients who experienced recurrence during follow-up (cases) and six individually matched patients who had not developed recurrence and were alive at the date of the case's recurrence (controls). Baseline characteristics of cases and controls are summarized in Table 1. Baseline characteristics for both groups were comparable in clinicopathological characteristics (tumor grade, lymph node status, and tumor size) and treatment received. Compared to controls, a higher proportion of cases (50%) had a body mass index > 25 kg/m^2, although not statistically significant.

Global methylation levels between cases and controls were not statistically different: the median M-values of cases were 0.487, and the median M-values of controls were 0.504 (P-value: 0.844). At probe methylation levels, we identified 2,009 CpGs (1,382 genes) that were differentially methylated between cases and controls: 1,200 DMPs (885 genes) were significantly hypermethylated and 809 DMPs (497 genes) were significantly hypomethylated in tumor tissues of cases compared to those of controls after multiple testing correction (FDR < 0.05).

Fifteen genes had a |log2FC| > 2.0: ten genes (*SIX2*, *PLEC1*, *ZNF833*, *RAI1*, *ZNF598*, *USP4*, *DOCK1*, *UNC84A*, *KLF16*, *PRKACA*) were significantly hypermethylated, and five genes [*STK33*, *TBXT* (alias *T*),

Table 1. Baseline characteristics of the whole study population, case group and control group

Factor	Whole study (*n* = 12)	Cases (*n* = 6)	Controls (*n* = 6)	*P*-value
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (years)	51.3 ± 5.6	51.3 ± 6.1	51.2 ± 5.6	0.87
Body mass index (kg/m^2)	23.8 ± 2.2	23.8 ± 2.6	23.9 ± 1.6	0.94
Follow-up time (years)	5.5 ± 2.9	3.8 ± 2.8	7.2 ± 2.1	0.04
	n (%)	*n* (%)	*n* (%)	
Grade				
I/II	3 (23%)	2 (33%)	1 (17%)	1.00
III	9 (77%)	4 (67%)	5 (83%)	
Lymph node status				
Negative	1 (8%)	0 (0%)	1 (17%)	1.00
Positive	11 (92%)	6 (100%)	5 (83%)	
Tumor size (cm)				
≤ 5	11 (92%)	5 (83%)	6 (100%)	1.00
> 5	1 (8%)	1 (17%)	0 (0%)	
Estrogen receptor status				
Negative	4 (33%)	2 (33%)	2 (33%)	1.00
Positive	8 (67%)	4 (67%)	4 (67%)	
Progesterone receptor status				
Negative	6 (50%)	3 (50%)	3 (50%)	1.00
Positive	6 (50%)	3 (50%)	3 (50%)	
Menopausal status				
Pre	4 (33%)	2 (33%)	2 (33%)	1.00
Post	8 (67%)	4 (67%)	4 (67%)	
Radiotherapy				
No	1 (8%)	1 (17%)	0 (0%)	1.00
Yes	11 (92%)	5 (83%)	6 (100%)	
Endocrine therapy				
No	4 (33%)	2 (33%)	2 (33%)	1.00
Yes	8 (67%)	4 (67%)	4 (67%)	
Chemotherapy				
No	0 (0%)	0 (0%)	0 (0%)	1.00
Yes	12 (100%)	6 (100%)	6 (100%)	
Trastuzumab treatment completed				
No	0 (0%)	0 (0%)	0 (0%)	1.00
Yes	12 (100%)	6 (100%)	6 (100%)	

n: Number of subjects; SD: standard deviation.

KCNH7, *ADAMTS2*, *FAM19A5*] were significantly hypomethylated in breast cancer tissues of cases compared to controls. Results are reported in Table 2.

Systematic review of published findings

Study selection

Of the 758 references retrieved by electronic search in PubMed, we reviewed 52 full-text documents, and fifteen met the eligibility criteria [Figure 1]. This review also included our pilot study that assessed the association between DNA methylation patterns in breast cancer specimens and response to trastuzumab in a cohort of 12 HER2-positive breast cancer patients.

Study characteristics

In all selected studies, epigenetic and genetic patterns were measured genome-wide in breast cancer tissues or blood. DNA methylation patterns were evaluated in one study (our pilot study), gene expression profile in nine studies[45-53], miRNA expression in two studies[54,55], long intergenic non-coding RNA (lincRNA) profile in one study[50], copy number alteration (CNA) profile in two studies[48,56], protein expression in one study[57] and mutations in two studies[58,59]. One study reported both genome-wide gene expression and

Table 2. Genes differentially methylated in breast cancer tissues of cases compared to controls

CpG sites	Chr	Gene	Gene region	CpG island region	LogFC	q-value
cg08788717	chr11	STK33	TSS200	Island	-2.835	0.036
cg02149708	chr6	TBXT (T)	TSS200	Island	-2.384	0.038
cg26974327	chr2	KCNH7	TSS200	OpenSea	-2.150	0.037
cg05214690	chr5	ADAMTS2	1stExon	Island	-2.127	0.015
cg22643811	chr22	FAM19A5 (TAFA5)	1stExon	Island	-2.024	0.015
cg26391832	chr2	SIX2	TSS1500	S_Shore	2.042	0.028
cg21672292	chr8	PLEC1	Body	Island	2.097	0.039
cg26590664	chr19	ZNF833	TSS200	N_Shore	2.114	0.030
cg21771200	chr19	ZNF833	TSS200	N_Shore	2.270	0.028
cg02147681	chr17	RAI1	5'UTR	Island	2.235	0.035
cg03654304	chr16	ZNF598	Body	Island	2.287	0.038
cg18886444	chr3	USP4	TSS1500	S_Shore	2.366	0.038
cg26353296	chr3	USP4	TSS1500	S_Shore	2.381	0.034
cg06406458	chr10	DOCK1	Body	OpenSea	2.028	0.044
cg26987690	chr7	UNC84A	Body	S_Shore	2.037	0.037
cg08287334	chr19	KLF16	Body	Island	2.230	0.049
cg19586199	chr19	PRKACA	TSS200; body	N_Shelf	2.582	0.030

Chr: Chromosome.

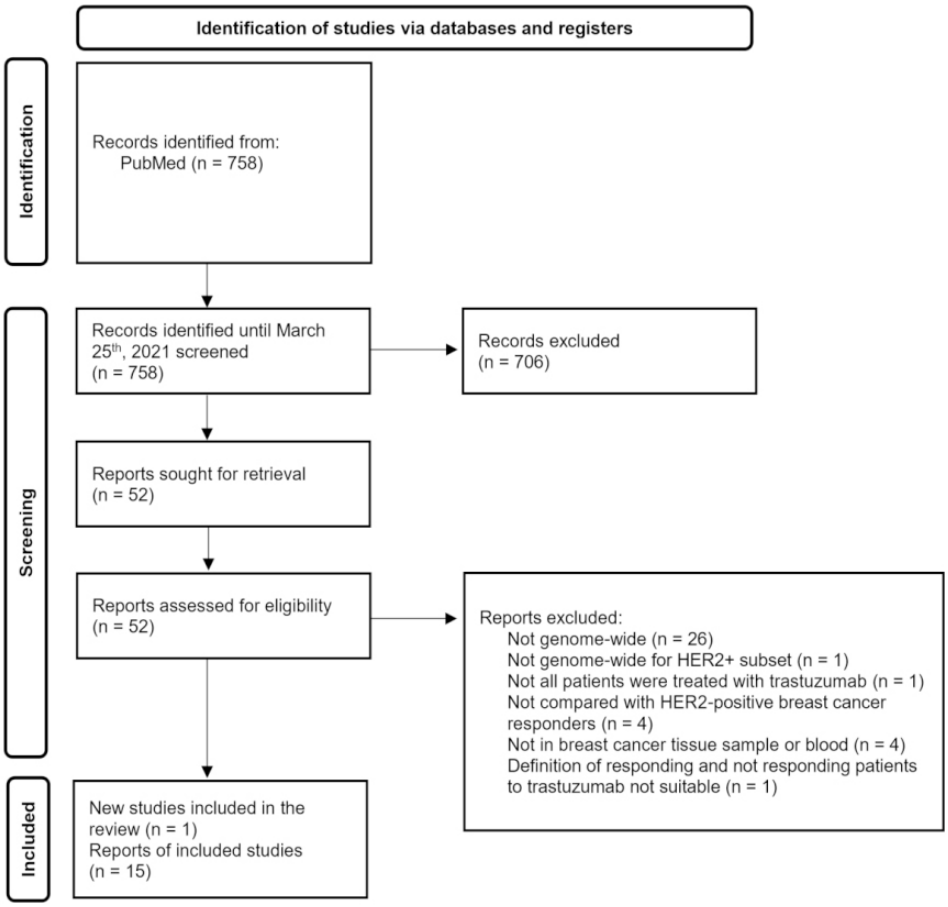

Figure 1. Process flow for article selection (PRISMA 2020 flow diagram).

genome-wide CNA patterns[48], and one reported both genome-wide gene and genome-wide lincRNA expression profiles[50]. The majority of studies were retrospective (n = 10)[46,47,49-51,54-57] including ours, and six studies were prospective[45,48,52,53,58,59] [Table 3].

DFS was reported in four studies including ours[46,52,54], RFS in two studies[49,53], and pCR in ten studies[45,47,48,50,51,55-59]. DFS was defined as the time between the diagnosis and recurrence (locoregional recurrence, recurrence in the contralateral breast, and distant breast cancer recurrence) or death; RFS was defined as the time from the start of trastuzumab treatment to the first local, regional or distant recurrence event; and pCR as the absence of invasive breast cancer in the breast and axillary lymph nodes at the time of surgery. Besides ours, the other fifteen included studies were published between 2007 and 2020 and involved between 9 and 849 HER2-positive, anti-HER2 therapy-treated breast cancer patients [Table 3].

Studies of genome-wide DNA methylation and response to targeted treatment

Characteristics of the study (our new findings) that evaluated the association between genome-wide DNA methylation patterns measured in breast cancer tissues and response to targeted treatment in HER2-positive, trastuzumab-treated breast cancer patients are presented in Supplementary Table 3. The study was retrospective. The mean age of included HER2-positive breast cancer patients was 51.3 years. The proportion of ER-positive breast cancer patients was 67%, and the proportion of positive lymph node status was 92%. Sixty-seven percent of HER2-positive breast cancer patients were postmenopausal. DNA methylation was assessed using Infinium HumanMethylation450 BeadChip array. Breast cancer patients were treated with adjuvant chemotherapy and trastuzumab. The study reported DFS. HER2-positive breast cancer patients were of Caucasian ethnicity. Tumor cell fraction was ≥ 70%.

Studies of genome-wide gene expression profile and response to targeted treatment

Characteristics of the nine studies that examined the association between genome-wide gene expression profiles measured in breast cancer tissues and response to HER2-targeted therapies in HER2-positive breast cancer patients are presented in Supplementary Table 4. Five studies were retrospective[46,47,49-51], and four studies were prospective[45,48,52,53]. The median age of HER2-positive breast cancer patients was not reported in four studies[46,48,50,51] and varied from 52 to 58 years in two studies[47,49]. The mean age was 55.2 years in one study[52], 60.9% of participants were < 45 years in another[45], and 48.3% of participants were < 50 years in one other study[53]. The proportion of ER-positive breast cancer patients was reported in three studies and varied between 43.4% and 69.6%[45,47,49]. In eight studies, patients received trastuzumab[45-47,49-53], and in the remaining study, patients were treated with trastuzumab, lapatinib or both[48]. pCR was reported in five studies[45,47,48,50,51], RFS in two studies[49,53], and DFS in two studies[46,52]. Gene expression profile was evaluated in fresh frozen tissue in five studies[45,46,48,50,51] and in FFPE tissues in four studies[47,49,52,53]. Gene expression profile was assessed using HumanHT-12 v3 BeadChip[46], HumanHT-12 v4 BeadChip[49], Affymetrix Human Genome U219 array[47], Affymetrix GeneChip 3' IVT Express kit[48], Illumina HiSeq 2500 platform[50], Illumina HiSeq 3000[52], GeneChip U133 Plus 2.0 Gene Array[45], DASL technology[53] and Agilent UNC Perou Lab Homo sapiens 1X44K Custom Array/Illumina HumanHT-12 WG-DASL V4.0 R2 expression BeadChip/Affymetrix technology using the HG-U133 Plus 2.0 GeneChip array[51]. Tumor cell fraction was not reported in five studies[47,48,50,51,53], and was at least 70% in three studies[46,49,52]. In the remaining study, breast cancer samples of each patient contained at least 500 breast cancer cells[45] [Supplementary Table 4].

Study of genome-wide miRNA expression profile and response to targeted treatment

Characteristics of the two studies that examined the association between genome-wide miRNA expression profile and response to targeted treatment in HER2-positive trastuzumab-treated breast cancer patients are presented in Supplementary Table 5. Both studies had a retrospective design[54,55]. miRNA expression was

Table 3. Summary characteristics of studies reporting on the association between genome-wide epigenetic and genetic modifications and response to HER2-targeted therapies in HER2-positive breast cancer patients (*n* = 16)

Study design	**Prospective studies:** *n* = 6 (Harris[45], Guarneri[48], Shi[59], Sorokin[52], Perez[53], Lesurf[58]) **Retrospective studies:** *n* = 10 (Khoury[46], Gámez-Pozo[47], Triulzi[49], Du[54], Merry[50], Ohzawa[55], Zhao[51], Yang[57], Walsh[56], Furrer [2022])
HER2-positive breast cancer patients	**Number of patients:** 9 to 849 **Age:** median - 50 to 59 y (*n* = 6) (Gámez-Pozo[47], Triulzi[49], Ohzawa[55], Walsh[56], Yang[57], Lesurf[58]); mean - 51.3 to 55.2 y (*n* = 2) (Furrer [2022], Sorokin[52]); < 45 y in 61% (*n* = 1) (Harris[45]); < 50 y in 48.3% (*n* = 1) (Perez[53]); < 65 y in 90.3% (*n* = 1) (Shi[59]); NR (*n* = 5) (Khoury[46], Merry[50], Guarneri[48], Zhao[51], Du[54]) **Stage:** Non metastatic I-III: *n* = 1 (Du[54]) Non metastatic II-III: *n* = 4 (Harris[45], Ohzawa[55], Sorokin[52], Furrer [2022]) Non metastatic NR: *n* = 3 (Guarneri[48], Triulzi[49], Perez[53]) Metastatic IV: *n* = 2 (Gámez-Pozo[47], Walsh[56]) Non metastatic and metastatic: *n* = 1 (Khoury[46]) NR: *n* = 5 (Merry[50], Shi[59], Zhao[51], Yang[57], Lesurf[58]) **Treatment received:** Neoadjuvant NVB and T: *n* = 1 (Harris[45]) Adjuvant T: *n* = 1 (Khoury[46]) Adjuvant or neoadjuvant CT (ATC-based, TAX-based, ATC + TAX, Other CT) and T: *n* = 1 (Gámez-Pozo[47]) Neoadjuvant CT (PTX and FEC) plus either T (arm A), L (arm B), or T + L (arm C): *n* = 1 (Guarneri[48]) Neoadjuvant PTX plus either T, L, or T + L: *n* = 1 (Shi[59]) Adjuvant CT and T: *n* = 4 (Triulzi[49], Du[54], Merry[50], Ohzawa[55]) Neoadjuvant CT and T: *n* = 2 (Zhao[51], Lesurf[58]) Adjuvant T only or T plus DTX/PTX + CBDCA/PTX/DTX + CBDCA/Cap/NVB/Gem: *n* = 1 (Sorokin[52]) Neoadjuvant therapy or surgical treatment and T: *n* = 1 (Yang[57]) T: *n* = 1 (Walsh[56]) Adjuvant ATC + CTX, PTX, T, or ATC + CTX, PTX + T, T: *n* = 1 (Perez[53])
Genome-wide profiling method	**Methylation:** *n* = 1 (Furrer [2022]) **miRNA expression:** *n* = 2 (Du[54], Ohzawa[55]) **Gene expression:** *n* = 9 (Harris[45], Khoury[46], Gámez-Pozo[47], Guarneri[48], Triulzi[49], Merry[50], Zhao[51], Sorokin[52], Perez[53]) **CNA:** *n* = 2 (Guarneri[48], Walsh[56]) **lincRNA expression:** *n* = 1 (Merry[50]) **Protein expression:** *n* = 1 (Yang[57]) **Mutations:** *n* = 2 (Lesurf[58], Shi[59])
Outcome	**Disease-free survival (DFS):** *n* = 4 (Khoury[46], Du[54], Sorokin[52], Furrer [2022]) **Relapse-free survival (RFS):** *n* = 2 (Perez[53], Triulzi[49]) **Pathological complete response (pCR):** *n* = 10 (Harris[45], Gámez-Pozo[47], Guarneri[48], Merry[50], Shi[59], Ohzawa[55], Zhao[51], Yang[57], Walsh[56], Lesurf[58])

ATC: Anthracycline; Cap: capecitabine; CBDCA: carboplatin; CNA: copy number alteration; CT: chemotherapy; CTX: cyclophosphamide; DTX: docetaxel; FEC: fluorouracil, epirubicin, and cyclophosphamide; Gem: gemcitabine; L: lapatinib; NVB: vinorelbine; NR: not reported; PTX: paclitaxel; T: trastuzumab; TAX: taxane; y: years.

measured genome-wide in FFPE breast cancer tissues using Agilent miRNA assay[54] or Agilent SurePrint G3 Human miRNA microarray[55] in cohorts of 14 and 40 HER2-positive breast cancer patients. Patients were treated with trastuzumab. DFS[54] and pCR[55] were reported. Tumor cell fraction was not reported in one study[54] and was at least 80% in the other[55].

Study of genome-wide lincRNA expression profile and response to targeted treatment

Characteristics of the single study that examined the association between genome-wide lincRNA expression profile and response to targeted treatment in HER2-positive trastuzumab-treated breast cancer patients are presented in Supplementary Table 6. The study had a retrospective design. lincRNA expression was measured genome-wide in fresh frozen breast cancer tissues using the Illumina HiSeq 2500 platform in a cohort of 13 HER2-positive breast cancer patients. Patients were treated with trastuzumab and pCR was reported. Tumor cell fraction was not reported.

Study of genome-wide CNA profile and response to targeted treatment

Characteristics of the two studies that examined the association between genome-wide CNA profile and response to HER2-targeted therapies in HER2-positive breast cancer patients are presented in Supplementary Table 7. The studies had a prospective design[48,56]. CNA profile was assessed genome-wide in fresh frozen breast cancer tissues using Affymetrix Genome-wide Human SNP array[48] or Illumina HiSeq[56] in cohorts of 68 and 11 HER2-positive breast cancer patients. Patients were treated with trastuzumab, lapatinib or both in one study[48] and trastuzumab in the other[56]. pCR was reported. Tumor cell fraction was not reported.

Study of genome-wide protein expression profile and response to targeted treatment

Characteristics of the only study that examined the association between genome-wide protein expression profile and response to targeted treatment in HER2-positive trastuzumab-treated breast cancer patients are presented in Supplementary Table 8. The study had a retrospective design[57]. The protein expression profile was assessed genome-widely in blood using TMT-6 plex Isobaric Label Reagent Set in a cohort of 6 HER2-positive breast cancer patients. Patients were treated with trastuzumab. pCR was reported.

Study of genome-wide mutation profile and response to targeted treatment

Characteristics of the two studies that examined the association between genome-wide somatic or germline mutation profile and response to targeted treatment in HER2-positive breast cancer patients are presented in Supplementary Table 9. The studies had a prospective design[58,59]. Somatic mutation profile was assessed genome-wide in fresh frozen breast cancer tissues for the two studies. Germline mutation profile was assessed genome-widely in blood using Illumina HiSeq 2000[59], and whole genome sequencing (WGS) and whole exome sequencing (WES) + Illumina HiSeq 2000 platform[58] in cohorts of 48 and 203 HER2-positive breast cancer patients. Patients were treated with trastuzumab. pCR was reported. Tumor cell fraction was not reported in one study[58] and was at least 10% in the other study[59].

Risk of bias in studies

Overall, studies ranged on average from low to critical risk of bias [low (n = 2)[53,59], moderate (n = 4, including our study)[47,49,57], serious (n = 1)[54], and critical (n = 9)][45,46,48,50-52,55,56,58], most commonly due to confounding. The bias evaluation of each included study is presented in Supplementary Table 10.

Results of individual studies

Genome-wide gene expression profile in breast cancer tissues and response to anti-HER2 agents in HER2-positive breast cancer patients

The genes reported to be differentially expressed in breast cancer tissues of cases compared to controls for each identified study are presented in Supplementary Table 11. Higher expression of the following genes was consistently observed in two different studies: *ESR1*[49,50], *RBP1*, *SLP1*[46,50], *EPS8L1*[50,52], *MEP1B*, *UTP15*, *RRAS2*, *GRB10*, *FAM98A*, *RBMS2*, *RPL9*, *RPAIN*, *MORF4L1*, *LLPH*, *MTMR1*, *FRYL*, *FLCN*, *CLINT1*, *ERICH1*[47,52], *ZNF281*, *ANKRD52*, *PIAS3*, *BRD1*, *RNF146*, *CLDN12*, *PROM2*, *COMMD5*, *VMP1*, *DEDD*, *ZNF439*, *CRIP2*, *PRSS16*, *GUK1*, *RRN3*, *PLEKHG3*, *JUN*, *BCL9*, *SLC25A37*, *CRYZ*, *RNF24*, *PSMG3*, *PAQR7*, *ABT1*, *WNT7B*, *SLC35B2*, *SYTL4*, *NUPR1*, *DPY19L1*, *DAZAP1*, *EEF1D*, *SGPP1*, *GALNT2*, *SPA17*, *RAD51*, *MBD6*, *KIF1C*, *C1QTNF3*, *BLOC1S2*, *SLC2A10*, *ZNF740*, *ADCK2*, *SLC41A1*, *RAB4A*, *CRIP1*, *ZNF552*, *CARHSP1*, *POFUT1*, *EMC10*, *BAX*, *HOXC4*, *DDR1*, *CTSD*, *FEN1*, *SULT1A1*, *DUSP14*, *IRF9*, *TMC4*, *MUC1*, *LMAN2*, *LASP1*, *SHROOM1*[50,52], *NSL1*, *ENAH*, *UBE2Q2*, *GNPAT*, *THBS2*, *TBCEL*, *FAM46A*, *ZNF678*, *TSEN15*, *ZNF674*, *CNIH4*, *ASAH1*, *SELT*, *ARFGAP3*, *TATDN3*, *FBLN1*, *MOSPD1*, *PPCS*, *NUCKS1*, *PGBD2*, *ACBD3*, *ORMDL2*, *AMMECR1*, *TNFRSF19*, *FOSL2*, *PYCR2*, *WSB1*, *TROVE2*, *RWDD3*[47,50], *LYSMD3*[47,50], *APOB*, *SLC3A2*, *CST3*[52,57], *BOC*[45,52], *DDX27*, *IL17RC*, *PKP3*, *WNK2*[52,53] *AGRN*,

ATRN, *NACC1*, *PCSK6*, *PIR*, *PLAUR*, *QSOX1*, *RHBDL2*, *SEC22B*, *SERPINH1*, *TCEAL1*[50,53], *CSDE1*, *SCUBE3*, *TMEM167A*[47,53]. Lower expression of the following genes was consistently reported in two different studies: *ORMDL3*[49,50], *NME1*[46,50], *SLC35B1*[46,50], *UTP18*[46,50], *PHB*[46,50], *S100B*, *TOM1L1*[50,52], *HIST1H2BG*, *PRR13*[47,50], *NXPH1*, *CKS2*, *NETO2*, *SLC12A2*[46,52], *ACSL5*, *CD3E*, *GIMAP7*, *GZMK*, *PPARG*, *SELL*, *VCAM1*[50,53], *CLEC10A*, *CTLA4*, *FGL2*, *TSPAN7*[52,53], *DENND3*, *TIAM1*[47,53]. The following genes were reported to be higher expressed in breast cancer samples of cases compared to controls in two different studies but were reported to be lower expressed in another study: *SFRP1*[45,46,50], *ACTR1B*, *FASTK*, *TMEM219*, *NDUFA3*[47,50,52], *ATP5I*[47,50,52]. The following genes were reported to be lower expressed in breast cancer samples of cases compared to controls in two different studies but were reported to be higher expressed in another study: *PABPC1*[47,50,52], *ARPC1A*, *TXN*[47,50,52], *TOB1*[46,50,52]. Higher expression of the *GOLGA2*[47,50,52] and *PHF21A*[47,50,52] genes was consistently observed in three different studies.

Gámez-Pozo *et al.* and Sorokin *et al.* identified several pathways associated with response to trastuzumab, including those involved in EGF receptor signaling, PI3K, apoptosis signaling, and p53[47,52]. Gámez-Pozo *et al.* observed that the PI3K pathway was the most strongly associated with treatment response[47] [Supplementary Table 12]. Sorokin *et al.* identified several pathways associated with response to trastuzumab. The most statistically and significantly upregulated ones in the trastuzumab-sensitive group were PPAR Pathway and cAMP Protein Retention Pathway[52]. Triulzi *et al.* and Sorokin *et al.* reported that breast cancer samples of patients with a lower risk of early relapse showed higher expression of genes enriched in immune system-related pathways and proliferation-associated pathways[49,52]. For two[46,48] out of the four included studies that did not report pathway analysis[45,46,48,50], we performed gene ontology analysis using the list of genes reported as being differentially expressed by the authors using PANTHER. We observed that the Notch signaling pathway was overrepresented in both studies[46,48], an observation also reported by Sorokin *et al.*[52]. We observed that Wnt signaling was overexpressed in Khoury *et al.*[46], as reported by Sorokin *et al.*[52]. We did not perform pathway analysis for the two remaining studies[45,53], as the number of differentially expressed genes (n = 11) was too small to perform the analysis[45] or not reported[53].

In our study, we observed overlap between the identified differentially expressed genes and the strongly differentially methylated (i.e., $|\log2FC| > 2.0$) genes. *PRKACA* was hypermethylated in our study (within the TSS region as well as the gene body), upregulated in one study[50], and downregulated in another study[47].

Genome-wide miRNA expression profile in breast cancer tissues and response to trastuzumab in HER2-positive breast cancer patients treated with trastuzumab

Du *et al.* identified seven upregulated and two downregulated miRNAs in breast cancer tissues of cases compared to controls[54]. Ohzawa *et al.* identified four upregulated and ten downregulated miRNAs in breast cancer tissues of cases compared to controls[55] [Supplementary Table 13]. Regarding the miRNAs identified by Du *et al.*, 902 genes were predicted to be targeted by miR-150-5p, 47 genes by miR-4734, 570 genes by miR-361-5p, 1,134 genes by miR-26a-5p, 416 genes by miR-365a-3p, 701 genes by miR-155-5p, 737 genes by miR-205-5p, 1,384 genes by miR-106b-5p, and 187 genes by miR-424-3p (as illustrated in a database for miRNA target prediction and functional annotations available online at www.mirdb.org)[54]. For the miRNAs reported by Ohzawa *et al.*, 484 genes were predicted to be targeted by miR-210, 242 genes by miR-31-3p, 891 genes by miR-449a, 801 genes by miR-449b-5p, 21 genes by miR-106b-3p, 263 genes by miR-1180, 242 genes by miR-1238-5p, 1,133 genes by miR-142-5p, 902 genes by miR-150-5p, 1,409 genes by miR-181c-5p, 1,266 genes by miR-182-5p, 438 genes by miR-20a-5p, 1,084 genes by miR-218-5p, 1,249 genes by miR-3609, 270 genes by miR-362-5p, 420 genes by miR-3620-3p, 676 genes by miR-4418, 272 genes by miR-4506, 410 genes by miR-4657, 406 genes by miR-505-3p, and 392 genes by miR-505-5p[55].

We observed that among the 344 genes that were reported to be differentially expressed (at the mRNA level) between breast cancer tissues of cases and controls in at least two studies, 170 were predicted to be targeted by miRNA identified as differentially expressed between breast cancer tissues of cases and controls in Ohzawa *et al.*[55] or Du *et al.*[54] [Supplementary Table 14]. We observed that among the 15 genes identified as differentially methylated in our study, five (*KCNH7*, *ADAMTS2*, *SIX2*, *DOCK1* and *ZNF598*) were predicted to be targeted by miRNA identified as differentially expressed in breast cancer tissues of cases compared to controls in the study of Ohzawa *et al.*[55] or Du *et al.*[54] [Supplementary Table 14].

Genome-wide lincRNA expression profile in breast cancer tissues and response to targeted treatment in HER2-positive breast cancer patients treated with trastuzumab

Merry and collaborators observed that 371 lincRNAs were differentially expressed in non-pCR samples compared to pCR samples, where 33 lincRNAs showed decreased expression and 338 increased expression[50] [Supplementary Table 15]. Among these 371 genes, 97 were reported to be differentially expressed at the mRNA level in breast cancer tissues of cases compared to controls in at least two studies: *FAM84B*, *PHF21A*, *NDUFV3*, *COMMD6*, *SRP9*, *S100B*, *WDR26*, *LYSMD3*, *CSTB*, *C5orf39*, *FOXA1*, *GALM*, *ITGB2*, *SP140*, *UTRN*, *SAA2*, *SHB*, *ZFP64*, *ZC3H12B*, *NADSYN1*, *B4GALNT4*, *AP2B1*, *USP16*, *ARL4D*, *SYNPO2*, *FAIM3*, *CBR1*, *EFR3B*, *IL18*, *LOC389493*, *FBXO16*, *FAM114A1*, *MAP3K9*, *TOX3*, *ZNF681*, *IDH3B*, *TPBG*, *PRKACB*, *UBE2A*, *ID2*, *IRX3*, *CILP*, *COL5A2*, *WRB*, *MBOAT1*, *GCA*, *SATB2*, *HERC6*, *RALGPS2*, *NUF2*, *MIA3*, *FAM91A1*, *FAM5C*, *C1orf227*, *RPP4*, *MYOT*, *PRKAA2*, *MAP1LC3B*, *PEX3*, *MEST*, *F13A1*, *CREB1*, *LRCH2*, *PCF11*, *POLR3G*, *RORA*, USP3, TSHZ3, CXCR4, *CCNH*, *CCM2*, *ZNF814*, *RAPH1*, *ZPBP2*, *FBXW4*, *ODF3B*, *CROCC*, S H 3 R F 2 , *HEATR6*, *CDK13*, *ATF3-1*, *ATF3*, *DTL*, *IARS2*, R C 3 H 1 , RC3H1, *URB2*, *RHOU*, *RC3H1*, *RC3H1*, *SUPT3H*, *ABI1*, *OTUD7B*, *GPATCH2*, *RNF2*, *IRF2BP2*.

Among the differentially expressed lincRNAs reported by Merry *et al.*[50], we observed 44 genes that were predicted to be target genes of miRNA identified as differentially expressed in the study of Ohzawa *et al.*[55] or Du *et al.*[54] [Supplementary Table 14].

None of the genes whose lincRNAs were reported to be differentially expressed between patients showing pCR and those showing non-pCR in Merry *et al.* was reported as differentially methylated with a $|\log_2 FC| > 2.0$ between cases and controls in our study[50]. However, when we consider the entire list of differentially methylated genes in our study, regardless of $\log_2$-fold change, we identified six overlapping genes. Among these six genes, five were hypermethylated (*GABRA5*, *ZIC5*, *GRAMD4*, *RSPH3*, and *VCAN*), and one was hypomethylated (*CSMD1*) in breast cancer samples of cases compared to controls.

Genome-wide copy number alterations in breast cancer tissue and response to targeted treatment in HER2-positive breast cancer patients treated with anti-HER2 agents

In their analysis of the association of genome-wide CNA in breast cancer tissues of HER2-positive breast cancer patients who received trastuzumab, lapatinib, or both, with response to anti-HER2 treatment, Guarneri *et al.* reported that, unlike pCR patients, non-pCR patients showed a CNA signature[48]. Overall, the authors observed CN alterations, mainly CN gains, in 557 genes located on chromosomes 1, 8, 17, 20 [Supplementary Table 16]. We observed that among the 344 genes that were reported to be differentially expressed in breast cancer tissues of cases compared to controls in at least two studies, 23 genes showed CN alterations in non-pCR samples compared to pCR-samples in Guarneri *et al.*[48]: *FAM84B*[46,48], *SRP9*[50,52], *WDR26*[47,50], *BATF3*[47,50], *EDARADD*, *ENPP2*, *LAX1*, *NUAK2*, *PTPRC*[53], *KIF21B*, *RNF19A*, *ZNF831*, *NR5A2*[52,53], *KIF26B*, *LAMB3*, *C1orf133*[50,53], *LAMC2*[52,53], *FAIM3*[50], *MIA3*[47], *OTUD7B*, *GPATCH2*, *RNF2*, *IRF2BP2*[50].

We observed that among the 557 genes that were reported to show CN alteration in non-pCR samples compared to pCR-samples in the study of Guarneri *et al.*[48], 279 were predicted to be targeted by miRNA identified as differentially expressed in breast cancer tissues of cases compared to controls in the study of Du *et al.*[54] or Ohzawa *et al.*[55] [Supplementary Table 14].

Among the genes showing CN alterations in non-pCR samples compared to pCR-samples in the study of Guarneri *et al.*[48], 18 genes (*FAM84B*, *SRP9*, *WDR26*, *MIA3*, *FAM91A1*, *FAM5C*, *C1orf227*, *ATF3*, *DTL*, *IARS2*, *RC3H1*, *URB2*, *RHOU*, *RC3H1*, *OTUD7B*, *GPATCH2*, *RNF2*, *IRF2BP2*) showed higher lincRNA expression and one (*FAIM3)* showed lower lincRNA expression in Merry *et al.*[50].

No overlap was observed between genes showing CN alterations in non-pCR samples compared to pCR-samples in Guarneri *et al.* and genes identified as differentially methylated with a $|\log2FC| > 2.0$ in our study[48]. However, when we consider the whole list of differentially methylated genes, we identified 27 genes that were differentially methylated in breast cancer tissues of cases compared to controls in our study among the 557 genes showing CNV variations in the study of Guarneri *et al.*[48]. Of these 27 differentially methylated genes, nine were hypomethylated (*PLD5*, *GJD2*, *C20orf85*, *APCDD1L*, *VASH2*, *PSEN2*, *FMN2*, *EDN3*, *ACTN2*) and 18 were hypermethylated (*TRAF5*, *TSEN15*, *TRIB1*, *TMEM206*, *SNX31*, *SMG7*, *RGS1*, *PRG4*, *PGBD5*, *NID1*, *KCNV1*, *GPATCH2*, *EXT1*, *CDK18*, *CAPN9*, *ADSS*, *ABR*, *C1orf55*).

Genome-wide protein expression profile in blood of breast cancer cases compared to controls

Out of the 18 genes that were reported as differentially expressed (five downregulated and 13 upregulated) in the blood of breast cancer cases compared to controls by Yang *et al.*[57] [Supplementary Table 17], three (*APOB*, *SLC3A2*, *CST3*) were differentially expressed in breast cancer tissues of cases compared to control in at least two studies. Three (*LDHA*, *DBF4B*, and *MASP1*) were predicted to be targeted by miRNA identified as differentially expressed in breast cancer tissues of cases compared to controls in the study of Du and collaborators[54] or Ohzawa and collaborators[55] [Supplementary Table 14].

None of the differentially expressed genes in the study of Yang *et al.* were differentially methylated in our study[57].

Genome-wide somatic and germline mutations profile in breast cancer tissues of cases compared to controls

Whereas Shi and collaborators observed that higher somatic mutation frequency in the *PIK3CA* gene in the breast tissues of cases was associated with trastuzumab resistance[59], Lesurf *et al.* reported that no somatic or germline mutations were associated with response to trastuzumab in breast cancer tissues of cases compared to controls[58] [Supplementary Table 18].

DISCUSSION

In a cohort of 12 HER2-positive breast cancer patients treated with trastuzumab, interrogation of DNA methylation using the Infinium HumanMethylation450 BeadChip allowed identifying genes that were differentially methylated between trastuzumab-resistant and trastuzumab-sensitive HER2-positive breast cancer patients. Interestingly, among the strongly differentially methylated genes, we observed genes associated with human cancer, including *DOCK1*, *ADAMTS2*, *PLEC1*, *USP4*, and *PRKACA*[60-69].

The guanine nucleotide exchange factor *DOCK1* (Dedicator of cytokinesis protein 1) is involved in cytoskeletal rearrangements required for phagocytosis of apoptotic cells and cell mobility[70]. A recent study reported that *DOCK1* inhibition leads to suppressed migration of the triple-negative breast cancer cell lines MDA-MB-157 and MDA-MB-231[68,71]. *ADMATS2* (ADAM metallopeptidase with thrombospondin type 1

motif 2) belongs to the ADAM metallopeptidase with thrombospondin type 1 motif and processes collagen precursors into mature collagen molecules[72]. It has been proposed that *ADAMTS2* exerts an anti-tumor effect by inhibiting intratumoral vascularization[73]. The *PLEC* gene encodes the pectin protein, which plays a role in maintaining tissue integrity[74]. A recent study suggests that a *PLEC* gene polymorphism (rs138924815) might increase the risk for familial testicular cancer[75]. *USP4* (Ubiquitin carboxyl-terminal hydrolase 4) is a deubiquitinating enzyme that removes conjugated ubiquitin from target proteins[76]. It has been reported that *USP4* expression was decreased in breast cancer tissue samples compared to paired normal breast tissues[63]. Moreover, *USP4* expression was associated with decreased proliferation in two HER2-negative breast cancer cell lines (MCF7 and BT549)[63]. The *PRKACA* gene encodes for a protein kinase that plays a role in controlling cellular processes such as glucose metabolism and cellular division[72]. Of note, one recent study suggests that *PRKACA* expression might be associated with the development of trastuzumab resistance in HER2-positive breast cancer patients[64]. The authors observed that in a subgroup of HER2-positive breast cancer patients who developed trastuzumab resistance (three out of five patients), *PRKACA* expression was highly increased in the breast cancer sample obtained after the onset of trastuzumab resistance compared to the pre-treatment sample. Considering that in our study, the *PRKACA* gene was hypermethylated within the gene body and that hypermethylation within this gene region often promotes gene elongation and, therefore, gene expression, we can postulate that our results might be concordant with those reported by Moody *et al.*[64]. Although in our study, DNA methylation was exclusively measured in pre-treatment samples and Moody *et al.* observed increased *PRKACA* expression only in breast cancer samples obtained after the onset of recurrence[64].

Among all genes that we identified as strongly differentially methylated between trastuzumab-resistant and trastuzumab-sensitive HER2-positive breast cancer patients, one of them, *PRKACA*, was reported as being higher expressed in breast cancer tissues of cases compared to controls in the study conducted by Merry and collaborators[50] and as being lower expressed in breast cancer tissues of cases compared to controls in the study of Gámez-Pozo *et al.*[47]. In our study, the *PRKACA* gene was hypermethylated within the TSS region and the gene body. Our observation could be partly concordant with the results reported by Merry *et al.*[50] (but not with those of Gámez-Pozo *et al.*[47]), as hypermethylation in the gene body (but not within the TSS region) is usually associated with increased gene expression[34]. Interestingly, the results of Merry and collaborators[50] (but not those of Gámez-Pozo *et al.*[47]) are concordant with the study above[64], where the authors observed that *PRKACA* expression was increased in breast cancer samples of HER2-positive trastuzumab-resistant breast cancer patients.

In one of the studies retained in our systematic review, the authors created a predictive model to differentiate HER2-positive trastuzumab-treated breast cancer patients with a higher risk of relapse from those with a lower risk in a cohort of 53 patients[49]. The validity of this predictive model was then confirmed in an independent and bigger data set. The authors observed that differentially expressed genes in the breast cancer tissues of patients identified as at low risk for relapse in the independent data set using this model were associated with the immune system. When we performed gene enrichment analysis of genes showing CN alteration in the study of Guarneri and collaborators, we observed that pathways associated with the immune response (inflammation mediated by chemokine and cytokine signaling pathway) were overrepresented. The immune system's involvement in response to anti-HER2 agents in HER2-positive breast cancer patients has also been reported in other studies[77-82].

To our knowledge, we conducted the first systematic review on the association of epigenetic and genetic alterations in breast cancer tissues or blood with the response to anti-HER2 agents in HER2-positive breast cancer patients. Sixteen studies were included in this review, and very few overlaps between studies were

found. The most consistent results were the higher expression of *GOLGA2* and *PHF21A* genes and the higher expression and CN gain of *MIA3*, *WDR26* and *C10rf133* genes observed in three different studies. Among these five genes, WDR26 has been shown to promote breast cancer growth and metastasis via the PI3K/AKT signaling pathway[83]. Gámez-Pozo and collaborators also observed that genes identified as differentially expressed in breast tumor tissues of cases compared to controls were overrepresented in the PI3K pathway[47]. Interestingly, the study by Shi and collaborators[59] also reported that PI3K mutations were associated with response to anti-HER2 agents in HER2-positive breast cancer patients and other previously published studies[84-86]. Taken together, these results suggest that genes of the PI3K pathway might play a relevant role in the development of resistance to anti-HER2 agents in breast cancer patients. Exploring the ramifications of these and other findings in larger cohorts or datasets like TCGA should be considered in future studies.

Several factors might explain why we only observed a few overlaps in our systematic review. Tumor cell content varied from > 40% to > 80% between studies, and in several publications (n = 4), this information was not provided. Contamination with cell types other than breast cancer cells can modify the observed pattern of genetic and epigenetic markers, as DNA methylation and other epigenetic or genetic markers widely vary across tissues and cellular types[87]. Moreover, the type of outcome evaluated in the study might also play a role, as the assessment of pCR in the neoadjuvant setting might mainly identify patients who did not primarily respond to targeted treatment (primary resistance), whereas the evaluation of DFS in the adjuvant setting might allow identifying patients who initially respond to targeted treatment but who develop resistance over time (acquired resistance). Moreover, epigenetic and genetic markers can be influenced by clinicopathological data, including age, stage, ER status, menopausal status, and ethnicity[88-96]. Unfortunately, it was difficult to evaluate this aspect, as patients' clinicopathological data were not extensively reported in the majority of the included studies. The risk of bias in most studies was due to confounding.

CONCLUSION

In conclusion, although the sample size of the present pilot study was small and despite the lack of validation cohort, using a high-throughput analysis, we identified genes that were differentially methylated in breast cancer tissues of HER2-positive trastuzumab-treated breast cancer patients who developed resistance toward this drug compared to those who responded to targeted therapy. One of the most differentially methylated genes, *PRKACA*, has been reported to be differentially expressed in breast cancer tissues of trastuzumab-resistant compared to trastuzumab-sensitive HER2-positive breast cancer patients in two studies included in our systematic review. Although we identified very few genes that overlap between studies, our review suggests that some of the genes acting in the PI3K pathway, such as *PRKACA*[97], might play an important role in developing resistance to anti-HER2 agents in breast cancer patients. Although the associations between *PI3KCA* mutations and *PI3K* dysfunctions and anti-HER2 treatment resistance are well documented in the literature[86,98-100], further studies on this topic are needed, which may help to unveil carcinogenic mechanisms involved in this pathway.

Although the observations reported in the retained studies were only marginally concordant, our work and the studies presented in this article suggest that knowledge gathered from these high-throughput studies could be useful for the identification of novel biomarkers of trastuzumab resistance. This might promote the development of new targeted drugs that could be administered to trastuzumab-resistant HER2-positive breast cancer patients.

DECLARATIONS

Authors' contributions

Conceived, acquired, analyzed and interpreted the data, wrote the original draft: Furrer D, Dragic D
Analyzed the data, edited the manuscript: Fournier F, Droit A, Jacob S
Reviewed and edited the manuscript: Chang SL
Conceived and designed the study, acquired, analyzed and interpreted the data, wrote and edited the manuscript, supervised and acquired funding for the study: Diorio C
All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Ethical approval of the study was obtained from the Research Ethics Committee of the Centre de Recherche du CHU de Québec (# 2016-2802).

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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The role of antiangiogenic monoclonal antibodies combined to EGFR-TKIs in the treatment of advanced non-small cell lung cancer with activating EGFR mutations: acquired resistance mechanisms and strategies to overcome them

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Abstract

As of today, only two antiangiogenic monoclonal antibodies plus epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) combinations are FDA and EMA-approved and are recommended by American Society of Clinical Oncology, European Society for Medical Oncology, and National Comprehensive Cancer Network for the first-line treatment of EGFR+ advanced non-small cell lung cancer patients: erlotinib plus bevacizumab and erlotinib plus ramucirumab. However, all treated patients eventually become unresponsive to such drugs, due to several different acquired resistance mechanisms, mainly represented by T790M substitutions and MET amplifications. While osimertinib treatment in T790M+ patients still represents the only approved treatment, MET-TKIs will likely change this status quo in the near future. In fact, existing clinical data strongly support a role for MET-TKI-based combinations in EGFR+ MET-amplified patients, possibly revolutionizing our current treatment algorithm. Chemotherapy plus immunotherapy plus antiangiogenic therapy combinations could also represent another useful addition.

Keywords: NSCLC, EGFR, T790M, MET, bevacizumab, ramucirumab, TKI, resistance mechanisms

INTRODUCTION

Epidemiology of EGFR+ NSCLC and preclinical background for antiangiogenic monoclonal antibody plus EGFR-TKI combinations

According to the most recent GLOBOCAN data, in 2020, lung cancer accounted for approximately 2,200,000 new cases and 1,800,000 deaths[1]. Overall, 85% of these cases are represented by non-small cell lung cancer (NSCLC), and the majority of NSCLC are histologically represented by adenocarcinomas (55%)[2]. With respect to genetic alterations, the vast majority of currently druggable mutations are diagnosed in adenocarcinomas, and specifically EGFR (epidermal growth factor receptor)-activating mutations (exon 19 deletion and exon 21 L858R substitution) are among the most common ones, accounting for approximately 15% of non-squamous NSCLC cases in North American and European patients and 40%-50% of non-squamous NSCLC in Asian patients[3].

With reference to EGFR mutations, vast literature data emphasize the link between EGFR axis hyperactivity and VEGF (vascular endothelial growth factor) induction and upregulation, as well as VEGFR (vascular endothelial growth factor receptor)-EGFR synergy, in promoting tumor growth, thus providing a strong preclinical background for the potential benefits of antiangiogenic monoclonal antibody plus EGFR-tyrosine kinase inhibitor combinations in this subset of patients[4-6].

CURRENT AND FUTURE CLINICAL PERSPECTIVES

Regulatory approvals and current guidelines involving antiangiogenic monoclonal antibody plus EGFR-TKI combinations

As of today, only two antiangiogenic monoclonal antibodies plus EGFR-TKI (tyrosine kinase inhibitor) combinations are FDA (US Food and Drug Administration) and EMA (European Medicines Agency) approved and ASCO (American Society of Clinical Oncology), ESMO (European Society for Medical Oncology), and NCCN (National Comprehensive Cancer Network) recommended for the first-line treatment of EGFR+ advanced NSCLC patients: erlotinib (EGFR TKI) plus bevacizumab (anti-VEGF-A mAb) and erlotinib plus ramucirumab (anti-VEGFR2 mAb)[7-10].

Pivotal trials involving antiangiogenic monoclonal antibody plus EGFR-TKI combinations

The erlotinib plus bevacizumab combination received regulatory approval and clinical recommendation thanks to the results coming from several different phase II and III trials. In the single-arm phase II BELIEF trial, 109 European naïve non-squamous advanced NSCLC patients with EGFR activating mutations were administered an erlotinib plus bevacizumab combination, achieving an overall PFS (progression-free survival) of 13.2 months and a manageable safety and tolerability profile with grade 4 TRAEs (treatment-related adverse events) in 4% of treated patients[11].

In the randomized controlled phase II JO25567 study, 154 Japanese naïve non-squamous advanced NSCLC patients with EGFR activating mutations were randomized 1:1 to receive erlotinib plus bevacizumab or erlotinib as monotherapy. The combination arm showed superior results with mPFS of 16.0 months *vs.* 9.7 months and HR (hazard ratio) for progression or death of 0.54 and comparable safety and tolerability profile with serious TRAEs in 24% of treated patients *vs.* 25% of treated patients[12]. However, after an extended follow-up of 34.7 months, while the combination arm continued to show superior results in terms of PFS at 16.4 months *vs.* 9.8 months (HR for progression or death, 0.52), no significant benefit in terms of OS (overall survival) was observed at 47.0 months *vs.* 47.4 months (HR for death, 0.81; $P = 0.3267$), and no new safety issues were reported[13].

Similarly, in the randomized controlled phase III NEJ026 trial, 228 Japanese naïve non-squamous advanced NSCLC patients with EGFR activating mutations were randomized 1:1 to be given erlotinib plus bevacizumab or erlotinib alone. At a first analysis, after a median follow-up of 12.4 months, the PFS results favored the combination arm at 16.9 months *vs.* 13.3 months (HR for progression or death, 0.60), and a manageable safety profile was observed with serious TRAES in 8% of treated patients *vs.* 4% of treated patients[14]. However, once again, at a longer follow-up of 39.2 months, no significant benefit in terms of OS was reported between the combination and monotherapy arms at 50.7 months *vs.* 46.2 months (HR for death, 1.007; $P = 0.97$)[15].

More recently, these conclusions have also been supported by the BEVERLY study. In the phase III randomized controlled BEVERLY trial, 160 Italian naïve advanced NSCLC patients with EGFR activating mutations were randomized 1:1 to receive erlotinib plus bevacizumab or erlotinib as monotherapy. After 31 months of median follow-up, a clear advantage was reported with respect to PFS at 15.4 months *vs.* 9.7 months (HR for death or progression, 0.60) but not with respect to OS at 28.4 months *vs.* 23.0 months (HR for death, 0.70; $P = 0.12$)[16,17].

Even more recently, the results from the randomized controlled phase III ARTEMIS-CTONG1509 trial also seem to be in line with the above-mentioned data. In this trial, 311 Chinese naïve advanced NSCLC patients with EGFR activating mutations were randomized 1:1 to be administered erlotinib plus bevacizumab or erlotinib alone. Once more, favorable PFS data were reported for the experimental arm at 17.9 months *vs.* 11.2 months (HR for death or progression, 0.55), but OS did not manage to reach significant superiority at 36.2 months *vs.* 31.6 months (HR for death, 0.92; $P = 0.581$)[18].

Ramucirumab received regulatory approval and clinical recommendation thanks to the data from the RELAY study. In this randomized controlled phase III trial, 449 European and Asian naïve advanced NSCLC patients with EGFR activating mutations were randomized 1:1 to receive erlotinib plus ramucirumab or erlotinib as monotherapy. At the pre-specified data cut-off after a median follow-up of 20.7 months, the combination arm performed better than the control one in terms of PFS at 19.4 months *vs.* 12.4 months (HR for death or progression, 0.59), albeit with some safety concerns with grade 3-4 TRAEs in 72% of treated patients *vs.* 54% of treated patients. OS data are still awaited[19-21]. In addition, following subgroup analysis, extremely interesting results were reported about TP53- and L858R-mutated patients. While EGFR+ TP53+ patients are classically associated with a worse outcome[22], they benefited from the experimental combination in terms of mPFS at 15.2 months *vs.* 10.6 months (HR for death or progression, 0.54); in particular, the HR data prove to be superior to those of ITT (0.59)[23]. Similarly, the L858R patients presented particularly favorable mPFS results at 19.4 months *vs.* 11.2 months (HR for death or progression, 0.618)[24]. It is worth mentioning that this result is superior to the one reported in the FLAURA trial (osimertinib *vs.* gefitinib or erlotinib in naïve EGFR+ advanced NSCLC patients) at 18.9 months *vs.* 10.2 months[25]. These results are particularly relevant because, while osimertinib (a third-generation EGFR-TKI) represents the most used and effective treatment in naïve EGFR+ advanced NSCLC patients, erlotinib plus ramucirumab combination could represent a more useful and attractive choice in these two selected subsets of patients. To further investigate this matter, a phase III trial comparing erlotinib plus ramucirumab to osimertinib monotherapy in naïve EGFR+ L858R advanced NSCLC patients is currently ongoing[26].

Acquired resistance mechanisms to antiangiogenic monoclonal antibody plus EGFR-TKI combinations

As the above-mentioned data extensively show, antiangiogenic monoclonal antibody plus EGFR-TKI combinations have managed to grant excellent results in terms of survival and safety, comparable with those of EGFR-TKI monotherapy [Table 1]. This is especially true when we compare these results with those

Table 1. Overview of data from trials exploring approved antiangiogenic mAbs plus EGFR-TKI combinations for the treatment of aNSCLC with activating EGFR mutations

Name of the trial (number of patients)	Phase of the trial	Experimental arm	Control arm	Efficacy data in months
BELIEF (*n* = 109)	II	Erlotinib + bevacizumab	/	mPFS: 13.2
JO25567 (*n* = 154)	II	Erlotinib + bevacizumab	Erlotinib	mPFS: 16.4 *vs.* 9.8 (HR: 0.54) mOS: 47.0 *vs.* 47.4 (HR: 0.81; *P* = 0.3267)
NEJ026 (*n* = 228)	III	Erlotinib + bevacizumab	Erlotinib	mPFS: 16.9 *vs.* 13.3 (HR: 0.60) mOS: 50.7 *vs.* 46.2 (HR: 1.007; *P* = 0.97)
BEVERLY (*n* = 160)	III	Erlotinib + bevacizumab	Erlotinib	mPFS: 15.4 *vs.* 9.7 (HR: 0.60) mOS: 28.4 *vs.* 23.0 (HR: 0.70; *P* = 0.12)
ARTEMIS-CTONG1509 (*n* = 311)	III	Erlotinib + bevacizumab	Erlotinib	mPFS: 17.9 *vs.* 11.2 (HR: 0.55) mOS: 36.2 *vs.* 31.6 (HR: 0.92; *P* = 0.581)
RELAY (*n* = 449)	III	Erlotinib + ramucirumab	Erlotinib	mPFS: 19.4 *vs.* 12.4 (HR: 0.59)

EGFR-TKI: Epidermal growth factor receptor-tyrosine kinase inhibitor; NSCLC: non-small cell lung cancer; HR: hazard ratio.

granted by standard chemotherapy and/or immunotherapy regimens[27-29]. However, even with these treatments, the progression of disease is inevitable. In fact, all the treated patients eventually become unresponsive to such drugs due to several different acquired resistance mechanisms that have been best investigated in patients treated with erlotinib with or without bevacizumab. Thanks to the vast literature data, it is now well established that the main acquired resistance mechanism in erlotinib-treated patients is represented by exon 20 T790M substitution (55%-60% of cases), followed by MET amplification (5), HER2 amplification (5%-10%), SCLC (small cell lung cancer) transformation (2%-10%), and epithelial-mesenchymal transition (2%-10%)[30-33].

The same applies to erlotinib plus bevacizumab-treated patients, albeit with different incidences. In fact, these patients seem to present lower rates of these mutations: T790M substitutions (35%-45% of cases), MET amplifications (3%-6%), HER2 amplification (2%-5%), and SCLC transformation (3%-5%)[18,34,35].

Current strategies and future perspectives in overcoming acquired resistance mechanisms

As of now, the only FDA- and EMA-approved and ASCO-, ESMO-, and NCCN-recommended treatment for EGFR+ advanced NSCLC patients progressing after EGFR-TKI therapy is osimertinib, the administration of which is limited to T790M+ patients. No approved agents are available for EGFR+ MET-amplified advanced NSCLC patients progressing after EGFR-TKI therapy, and platinum-based chemotherapy still represents the standard of care in this subset of patients[7-10].

Osimertinib received regulatory approval and clinical recommendation following the results from the AURA 3 trial. In this randomized controlled phase III trial, 419 EGFR+ T790M+ advanced NSCLC patients whose disease had progressed after first-line EGFR-TKI treatment were randomized 2:1 to receive osimertinib or cis/carboplatin plus pemetrexed followed by pemetrexed maintenance. At data cut-off, the osimertinib arm clearly outperformed the control one in terms of both response and survival with ORR (objective response rate) of 71% of treated patients *vs.* 31% of treated patients, PFS of 10.1 months *vs.* 4.4

months (HR for death or progression, 0.30) as well as in terms of safety and tolerability with grade ≥ 3 TRAEs in 23% of treated patients *vs.* 47% of treated patients[36]. However, in the final analysis, osimertinib did not show a significant benefit in terms of OS at 26.8 months *vs.* 22.5 months (HR for death, 0.87; P = 0.277). However, this result seems to be linked to the very high rate of crossover from platinum plus pemetrexed to osimertinib (73% of platinum-treated patients). In fact, very different results were reported after adjusting OS data for crossover: 26.8 months *vs.* 15.9 months (HR for death, 0.54)[37]. On a side note, it is worth mentioning that the main acquired resistance mechanism in patients progressing on second-line osimertinib is represented by MET amplification, accounting for 5%-50% of all cases[38]. The addition of bevacizumab to osimertinib failed to show meaningful PFS or OS improvement in both T790M+ pre-treated patients and naïve patients[39,40].

With respect to EGFR+ MET-amplified advanced NSCLC patients progressing after EGFR-TKI therapy, even though no drugs are approved yet, several encouraging trials have already assessed and are currently further investigating the safety and efficacy of different MET-TKIs, savolitinib, tepotinib, and capmatinib being the most promising ones[41]. In the phase Ib NCT02374645 trial, the savolitinib plus gefitinib (EGFR TKI) combination was administered to 57 Chinese EGFR+ MET-amplified advanced NSCLC patients progressing after EGFR-TKI therapy, reporting a manageable safety profile with grade ≥ 3 TRAEs in 27% of treated patients and reasonable ORR in T790M patients (52% of treated patients) and patients whose T790M state was unknown (40% of treated patients)[42].

Another savolitinib-based combination (osimertinib plus savolitinib) was investigated in the phase Ib TATTON trial. All 180 included patients were EGFR+ MET-amplified advanced NSCLC patients progressing after EGFR-TKI therapy. Patients in Cohort B1 were pre-treated with osimertinib; patients in Cohort B2 were not pre-treated with osimertinib and were T790M-; patients in Cohort B3 were not pre-treated with osimertinib and were T790M+; and patients in Cohort D were not pre-treated with osimertinib, but T790M-, and received a smaller dose of savolitinib. No dose-limiting toxicities were reported, 57% of patients in Cohort B and 38% of patients in Cohort D experienced grade 3 or worse TRAEs, and partial responses were reported in 48% of patients in Cohort B and 64% of patients in Cohort D[43].

The randomized controlled phase II NCT04606771 trial is currently recruiting EGFR+ MET-amplified advanced NSCLC patients progressing after osimertinib therapy to be randomized 1:1 to receive savolitinib plus osimertinib or savolitinib plus placebo. ORR is the primary objective, and the study completion date is estimated to be February 29, 2024[44].

Tepotinib was first investigated in combination with gefitinib in 55 Asian EGFR+ advanced NSCLC patients with acquired resistance to a previous EGFR-TKI (MET-amplification or overexpression) in the randomized phase Ib/II INSIGHT study. Even though this trial failed to show favorable survival results in the intention to treat population and was thus terminated early, extremely promising results were reported for the MET-amplified population: PFS of 16.6 months *vs.* 4.2 months (HR for death or progression, 0.13) and OS of 37.3 months *vs.* 13.1 months (HR for death, 0.08)[45]. These results were further confirmed in the final analysis: PFS of 19.3 months *vs.* 5.5 months (HR for death or progression, 0.18) and OS of 37.3 months *vs.* 13.1 months (HR for death, 0.08)[46]. Tepotinib with or without osimertinib is currently being investigated in the phase II INSIGHT 2 trial in EGFR+ MET-amplified advanced NSCLC patients progressing after osimertinib therapy. Recruitment is currently ongoing, dose-limiting toxicities and ORR are the primary objectives, and the study completion date is estimated to be March 30, 2023[47].

Table 2. Ongoing trials investigating MET-TKI-based combinations in advanced NSCLC patients

Clinical trial name	Phase	Subset of patients	Experimental arm	Control arm	Primary objective(s)	Study completion date
NCT04606771	II	EGFR+ MET-amplified progressing after osimertinib	Savolitinib + osimertinib	Savolitinib + placebo	ORR	February 29, 2024
INSIGHT 2	II	EGFR+ MET-amplified progressing after osimertinib	Tepotinib ± osimertinib	No control arm	Dose-limiting toxicities and ORR	March 30, 2023
GEOMETRY-E	III	EGFR+ MET-amplified T790M- progressing after EGFR TKI	Capmatinib + osimertinib	Cis/carboplatin + pemetrexed followed by a pemetrexed maintenance	Dose-limiting toxicities and PFS	March 30, 2027

MET-TKI: Mesenchymal epithelial transition-tyrosine kinase inhibitor; EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; ORR: objective response rate.

Finally, capmatinib was assessed in combination with gefitinib in the phase Ib/II NCT01610336 study, in which 161 EGFR+ advanced NSCLC patients with acquired resistance to a previous EGFR-TKI (MET-amplification or overexpression) received a capmatinib plus gefitinib combination therapy. Modest results were reported for the intention to treat the population with an ORR of 27%; however, remarkable results were reported for MET-amplified patients with an ORR of 47%[48,49]. The randomized controlled phase III GEOMETRY-E trial is currently recruiting EGFR+ MET-amplified T790M- advanced NSCLC patients progressing after EGFR-TKI treatment to be randomized 1:1 to receive a capmatinib plus osimertinib combination or cis/carboplatin plus pemetrexed followed by pemetrexed maintenance. Dose-limiting toxicities and PFS are the primary objectives and the study completion date is estimated to be March 30, 2027[50].

On a more general side note, it is worth mentioning that, in the recent IMpower150 trial, building on promising preclinical data[51], the carboplatin plus paclitaxel plus bevacizumab plus atezolizumab combination managed to be the first and currently only association of chemotherapy plus immunotherapy plus antiangiogenic therapy to show favorable results in EGFR-TKI-pretreated EGFR+ aNSCLC patients. In fact, when compared to the control arm (carboplatin plus paclitaxel plus bevacizumab), the former combination managed to outperform the latter with mOS of 27.8 months *vs.* 18.1 months (HR for death, 0.74)[52], thus representing another promising strategy in overcoming resistance mechanisms.

CONCLUSION

With reference to the main known acquired resistance mechanisms to the antiangiogenic monoclonal antibody plus EGFR-TKI combinations in EGFR+ advanced NSCLC patients (T790M substitution and MET amplification), we can report that, while osimertinib treatment in T790M+ patients still represents the only approved treatment, MET-TKIs will likely change this status quo in the near future. In fact, the extensive above-mentioned clinical data strongly support a role for MET-TKI-based combinations in EGFR+ MET-amplified patients, possibly revolutionizing our current treatment algorithm [Table 2]. Chemotherapy plus immunotherapy plus antiangiogenic therapy combinations could also represent another useful addition.

DECLARATIONS

Authors' contributions

All the authors contributed to the concept, design, draft and revision of this manuscript.

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Dr. Gridelli C received honoraria as speaker bureau and advisory board member from Astra Zeneca, BMS, MSD, Roche, Sanofi, GSK, Menarini, Karyopharm, Amgen, Pfizer, Eli Lilly, Takeda, Novartis; all the other authors have no conflicts of interest to declare.

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Cancer Drug Resistance

Review

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P-glycoprotein (ABCB1) - weak dipolar interactions provide the key to understanding allocrite recognition, binding, and transport

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Abstract

P-glycoprotein (ABCB1) is the first discovered mammalian member of the large family of ATP binding cassette (ABC) transporters. It facilitates the movement of compounds (called allocrites) across membranes, using the energy of ATP binding and hydrolysis. Here, we review the thermodynamics of allocrite binding and the kinetics of ATP hydrolysis by ABCB1. In combination with our previous molecular dynamics simulations, these data lead to a new model for allocrite transport by ABCB1. In contrast to previous models, we take into account that the transporter was evolutionarily optimized to operate within a membrane, which dictates the nature of interactions. Hydrophobic interactions drive lipid-water partitioning of allocrites, the transport process's first step. Weak dipolar interactions (including hydrogen bonding, π-π stacking, and π-cation interactions) drive allocrite recognition, binding, and transport by ABCB1 within the membrane. Increasing the lateral membrane packing density reduces allocrite partitioning but enhances dipolar interactions between allocrites and ABCB1. Allocrite flopping (or reorientation of the polar part towards the extracellular aqueous phase) occurs after hydrolysis of one ATP molecule and opening of ABCB1 at the extracellular side. Rebinding of ATP re-closes the transporter at the extracellular side and expels the potentially remaining allocrite into the membrane. The high sensitivity of the steady-state ATP hydrolysis rate to the nature and number of dipolar interactions, as well as to the dielectric constant of the membrane, points to a flopping process, which occurs to a large extent at the membrane-transporter interface. The proposed unidirectional ABCB1 transport cycle, driven by weak dipolar interactions, is consistent with membrane biophysics.

Keywords: Catalytic cycle, hydrogen bond, π-electron donor, polyspecificity, amphiphilicity, stoichiometry

INTRODUCTION

P-glycoprotein (ABCB1) (170 kDa) was discovered in 1976 by Juliano and Ling[1] in Chinese hamster ovary (CHO) cells, selected for resistance to colchicine. These cells displayed *pleiotropic cross-resistance* to a wide range of *amphiphilic* drugs. Because the glycoprotein altered the *membrane permeability (P)*, it was called P-glycoprotein. This first description comprises the key characteristics of the protein (highlighted in italics), which will play a recurrent role in this review. ABCB1 is the first mammalian member of a large family of ATP binding cassette (ABC) transporters present in prokaryotes[2] and eukaryotes, from plants[3] to humans[4]. Most ABC transporters move compounds (allocrites[5]) across membranes, using the energy of ATP binding and hydrolysis. The functional unit of ABC transporters consists of two highly conserved nucleotide-binding domains (NBDs) forming together two nucleotide-binding sites (NBSs) for ATP hydrolysis and two more variable transmembrane domains (TMDs), which are assumed to form the translocation pathway for allocrites. In prokaryotes, the functional unit consists of a homodimer formed by two polypeptides with one NBD and one TMD each. In most eukaryotes, these two polypeptides are linked and the transporters function as monomers. In recent years, approximately 250 structures of ABC transporters have been obtained, allowing the establishment of a new classification system based on TMD folds[6]. Prokaryotic ABC transporters revealed seven types of folds (I-VII), while eukaryotic ABC transporters had only two (IV and V). The seven subfamilies (ABCA-G), defined previously based on phylogenetic analysis[7], are maintained as subcategories within the type IV fold (subfamilies B-D) and type V fold (subfamilies A and G). Although the large number of structural approaches have provided important information, "the question as to how substrate binding and translocation are coordinated and coupled with ATP binding and hydrolysis, in any ABC transporter, remains elusive"[8,9].

Several models for ABCB1 function have been suggested. Senior and colleagues[10] observed that both NBSs of ABCB1 were catalytically active and seemed to alternate in activity, hydrolyzing one ATP per catalytic cycle with a turnover of 1-10 molecules s^{-1}. They suggested a scheme of alternating catalytic sites, in which drug transport is coupled to the relaxation of a high-energy catalytic site conformation, generated by the hydrolysis step[10]. Experiments by Sharom and colleagues[11] supported this model (for review, see[12]). More recently, one study demonstrated that ABCB1 "mutants with one intact catalytic center preserve the ability to hydrolyze ATP and to promote drug transport, suggesting that the two catalytic sites are randomly recruited for ATP hydrolysis"[13]. An analogous observation was made for ABCC7 (cystic fibrosis transmembrane conductance regulator, CFTR) that lacks the catalytic glutamine in NBD1[14]. Ambudkar and Sauna suggested that transport is coupled to the hydrolysis of a first ATP and resetting of the transporter to the hydrolysis of a second ATP[15].

On the basis of the first low-resolution structures of ABCB1 in the absence and presence of nucleotides[16], Higgins and Linton proposed the currently prevailing "ATP switch model"[17]. This model assumes that binding of two ATP molecules induces an outward-facing (OF) conformation for drug release, and hydrolysis of two ATP molecules leads to a nucleotide free (apo-form) with an inward-facing (IF) conformation for drug binding. Support for this switch, or alternating access model, was inferred from the OF high-resolution conformation of the homodimeric Sav1866 in the presence of two AMP-PNP molecules[18], and the IF conformation of apo-ABCB1[19,20]. An IF conformation of ABCB1 was also observed in permeabilized, ATP-depleted cells, in the presence of the conformation-sensitive antibody UIC2 mAb[13]. The resemblance of the alternating access model to the early "simple allosteric model for membrane pumps" by Jardetzky[21] proposed for moving inorganic ions across membranes was taken as further support for this model (for review, see e.g.,[22,23]).

The currently prevailing "alternating switch model" raises several questions. (i) Is the aqueous cleft of the apo-state, wide open to the cytosol, useful for the transport of amphiphilic compounds that highly accumulate in membranes and access the transporter within the membrane[24-27]? (ii) Is the assumption of an apo ground state realistic, considering the high intracellular ATP concentration (c_{ATP} = 1-10 mM)[28-30] and the comparatively low K_m values for ATP binding to the transporter (c_{ATP} = 0.4-0.8 mM)[31,32]? Are two ATPs hydrolyzed, or is one ATP hydrolyzed per transport cycle?

To obtain information on the conformation of the NBDs and TMDs under turnover conditions, we analyzed the results of multiple double electron-electron resonance (DEER) spectroscopy experiments performed with spin label pairs introduced at strategic locations in different ABC transporters and compared them with X-ray structures. The DEER experiments revealed a wide range of conformations that were not fully accounted for in the proposed models (see Ref.[33] and Supplementary Table 1 therein).

For further insight, we performed molecular dynamics (MD) simulations for different nucleotide occupancy states of Sav1866 structure, a prokaryotic ABCB1 homolog[33]. The two transporters (both with a type IV fold[6]) show overlapping substrate specificity[32,34]. Our simulations revealed an outward closed conformation of the transmembrane domain that is stabilized by the binding of two ATP molecules. The hydrolysis of a single ATP leads the X-loop, a key motif of the ATP binding cassette, to interfere with the transmembrane domain and favors its outward open conformation. These findings provided a structural basis for the unidirectionality of transport in ABC exporters and suggested that one ATP is hydrolyzed per transport cycle[33]. However, the role of the amphiphilic allocrites in the transport process remained unclear.

Here, we demonstrate that a quantitative understanding of the interactions between the amphiphilic allocrites and the transporter is possible if the membrane environment is taken into account. For this purpose, we review the thermodynamics (allocrite binding) and kinetics (ATP hydrolysis and allocrite transport rate) of ABCB1, published over approximately the past 25 years. Altogether, this work provides a compelling quantitative description of the nature of the intermolecular interactions relevant for ABCB1 function: Allocrite binding within the membrane, the rate of ATP hydrolysis, and allocrite transport are driven by weak dipolar forces that depend on the nature of the surrounding membrane. Combining the insights gained with the conclusions from our previous molecular dynamics simulations[33] generates a unidirectional allocrite transport cycle for ABCB1 that is consistent with the principles of membrane biophysics. Special emphasis is placed on clarifying the mechanism of ABCB1 inhibition, which is of crucial relevance for pharmacotherapy.

THE REACTION PARTNERS AND THEIR ENVIRONMENT

Understanding chemical processes generally starts with structural analysis of the reactants, in the present case, the ABCB1 transporter and its allocrites. Allocrites include substrates, modulators, and inhibitors (for definitions used, see Ref.[35]). Although less recognized, the environment in which the reaction partners meet determines the nature of interactions. Because ABCB1 and its allocrites meet in the membrane, we first provide a short description of the lipid bilayer and its properties relevant for the subsequent discussion of allocrite-transporter interactions (Supplementary Equation 1, discussed in detail below).

Characteristics of lipid membranes relevant for allocrite-transporter interactions

ABCB1 is abundant in plasma membranes. The plasma membrane shows an asymmetric lipid distribution between the bilayer leaflets. The extracellular leaflet is composed essentially of electrically neutral lipids

(phosphatidylcholines, sphingomyelins, and cholesterol). The cytosolic leaflet consists of electrically neutral phosphatidylethanolamines and anionic phosphatidylserines (for details, see[36,37]). The cytoplasmic leaflet is approximately twofold more unsaturated than the extracellular leaflet. These structural asymmetries are conserved throughout eukaryotes, suggesting fundamental cellular design principles[37].

The membrane is well described by its molecular order parameter, S_{mol}, the lateral packing density, π_M, the surface potential, Ψ_m, and the dielectric constant, ε_m. Each parameter is briefly explained below.

Molecular order parameter, S_{mol}, of lipid bilayers in the presence of "guest" molecules

Although membranes are highly organized, they are recalcitrant to crystallization because of considerable translational, rotational, and flexing movements of the constituent lipid and protein molecules. The dynamic "structure" of biological membranes is best characterized by deuterium nuclear magnetic resonance (^{2}H- or D-NMR) spectroscopy[38,39]. Chemically or biochemically exchanging protons with deuterons, either in the polar head group or in the acyl chains of lipids, provide information on the order and mobility of the molecules without disturbing the system. This is in contrast to most other labels including spin labels or fluorescent labels such as DPH (trimethylamine-diphenylhexatriene). Below, we give a few representative examples of how "guest" molecules (including cholesterol, peptides, membrane proteins, detergents, and drugs) influence the molecular order parameter of phospholipid bilayers.

Cholesterol

The addition of cholesterol to a dipalmitoylphosphatidylcholine (DPPC) bilayer in an equimolar amount doubles the order parameter of the fatty acyl chain region and eliminates the gel-to-liquid crystal phase transition (phase transition temperature for DPPC, T_m = 41 °C), producing a smooth order-temperature profile[40]. An enhanced order parameter was also reported for bilayers by 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC, with T_m = -2 °C)[41].

Peptides and proteins

The interaction of transmembrane proteins such as cytochrome c oxidase with the surrounding lipids has been investigated extensively by spin-label electron paramagnetic resonance (EPR) and NMR spectroscopy. EPR measurements (monitoring a high frequency range ~10^7-10^8 Hz) revealed two motionally distinct lipid populations. The term "boundary lipids" was coined for the slower component[42]. However, subsequent ^{2}H-, ^{31}P-, and ^{14}N-NMR investigations (monitoring a frequency range at least 10-fold lower) did not detect the presence of two lipid populations[43-45]. All lipids around the reconstituted cytochrome c oxidase exhibited very similar motional behavior and provided no evidence for motionally restricted boundary lipids, neither at the head group region nor at the cis-double bond[46]. To investigate potential interactions between cytochrome c oxidase and specific lipids, we characterized the few residual lipids attached to cytochrome c oxidase after delipidation using ^{31}P-NMR spectroscopy[47]. While most previous biochemical studies claimed that cardiolipin was the only lipid remaining after delipidation, the ^{31}P-NMR data show that all three lipids (phosphatidylethanolamine, phosphatidylcholine, and cardiolipin) can be observed, with cardiolipin being the least abundant. Compared with the effect of cholesterol, the effect of proteins and peptides on lipid membranes is thus generally almost negligible, indicating a perfect match between the movement of fluid-like hydrocarbon chains and the movement of the peptide side chains[48,49]. Natural cells also revealed rapid exchange of lipids around proteins on the NMR timescale (frequency range ~10^6 Hz)[50].

Detergents and drugs

Detergents (e.g.,[51,52]) and drugs (e.g.,[53]) have been well documented to cause membrane disorder at higher concentrations. Many detergents and drugs are allocrites for ABCB1. Interestingly, ABCB1 exports these

drugs and detergents well below the concentrations that lead to significant disorder of membranes (see below).

Mixing disparate lipids induces domain formation

Mixing bulky disordered lipids, such as the non-physiological DOPC or fluorescent lipids, with highly ordered lipids such as cholesterol and DPPC (e.g.,[54]) readily induces domain formation or phase separation. Hell and coworkers observed that, unlike phosphoglycerolipids, fluorescently labeled sphingolipids and glycosylphosphatidylinositol-anchored proteins can be transiently trapped (for approximately 10-20 ms) in cholesterol-mediated molecular complexes dwelling within < 20-nm-diameter areas[55].

Lateral membrane packing density, π_M

Generally, a higher order parameter S_{mol} is associated with higher lateral packing density of the membrane, π_M. A model membrane consisting of POPC, which is the most abundant lipid in mammalian membranes, exhibits a lateral packing density $\pi_M \approx 32$ mN/m at room temperature[56]. The addition of cholesterol (25 mol %) enhances the lateral packing density to $\pi_M \approx 35$ mN/m[57]. The lateral packing density, π_M, is relevant because it determines partitioning of compounds into the membrane[58]. To give an example embryonic cells (e.g., mouse embryo fibroblasts, NIH-3T3 cells[25]), with a low cholesterol content, exhibit lower lateral packing densities than adult mammalian cells[59].

Surface potential, Ψ_m

The cytoplasmic membrane leaflet exhibits a negative surface potential, Ψ_m. For mouse embryo fibroblasts (NIH-3T3 cells[25]), it was estimated as $\Psi_m \approx$ -30 to -20 mV under physiological conditions, assuming a cytosolic free magnesium concentration of C = 0.5 to 1 mM (with a membrane-binding constant for magnesium, $K = 10\ M^{-1}$) and a monovalent cation concentration of C = 100 to 150 mM (with a membrane-binding constant for monovalent cations, $K = 0.6\ M^{-1}$)[59]. Under the given conditions, a surface potential of $\Psi_m \approx$ -30 mV enhances the lipid-water partition coefficient, K_{lw}, of a strongly cationic drug by a factor of ~4. However, upon titration with cationic drugs, the surface potential decreases (see Ref.[32], Figure 7 therein).

Owing to the negative surface potential and the high unsaturation, the cytosolic plasma membrane leaflet acts as a drug scavenger for amphiphilic and cationic ABCB1 allocrites. The properties of the cytosolic membrane leaflet may even create a drug concentration gradient within the membrane that is opposed to the concentration gradient in the extracellular *vs.* intracellular aqueous phase. Notably, model membranes generally lack the asymmetry of natural membranes.

The dielectric constant, ε_m

Plasma membranes separate the extracellular and intracellular aqueous phases. Whereas the aqueous phase exhibits a high dielectric constant ($\varepsilon_m \approx 80$), the polar lipid head group regions of the membrane exhibit an intermediate one ($\varepsilon_m \approx$ 30-40) and the hydrophobic core region a very low one ($\varepsilon_m \approx$ 2-4)[60,61]. The dielectric constant, ε_m, decreases with increasing lateral membrane packing density, π_M, and thus varies somewhat with the lipid chain length, the degree of unsaturation, the cholesterol content, and the phase state of the membrane. The low dielectric constant, ε_m, of the membrane strengthens dipolar interactions.

Allocrite recognition

Long before ABCB1 structures were available, hundreds of allocrites had been identified. Allocrites are amphiphilic (or amphipathic)[1] and often cationic[62]. To explain the "polyspecificity" of ABCB1, we searched for recurrent elements in the chemical structures of drugs with the ability to interact with the

transporter *within* a lipid environment[24-27] (see also Ref.[63]). The analysis of a large number of chemical structures revealed specific patterns formed by Π-electron donor groups, that is, hydrogen bond acceptor groups (HBAs) and Π-electron systems (i.e., aromatic rings)[64,65] (see also[66,67]). The assumption that these patterns interact with the hydrogen bond donor groups (HBDs) and Π-electron systems (phenyl and tryptophan residues) in transmembrane helices of ABCB1 through dipolar interactions offers an explanation for the polyspecificity of ABCB1. Figure 1A-E display compounds with possible type I *and/or* type II patterns. Compounds with type II patterns are not only allocrites for ABCB1 but also inducers of ABCB1 expression by interacting with the nuclear pregnane X receptor, for example[68].

Notably, HBDs in allocrites (e.g., -OH, -NH_2, and > NH) do *not* interact with ABCB1[64]. However, they significantly reduce the lipid-water partition coefficient (e.g., Refs.[48,69]) and the rate of passive diffusion across the lipid membrane, which in turn enhances the risk of drugs being caught by ABCB1[58].

The transporter exposes multiple HBDs

Figure 2 (and Supplementary Figure 1A-D) displays the numerous HBDs in the transmembrane domain of apo-Abcb1a[20] and the nucleotide-bound ABCB1 (modeled on the high-resolution structure of Sav1866[18]) from side [Figure 2A and B] and top views [Figure 2C and D]. The amino acids with hydrogen bond donor groups are highlighted in green. Interestingly, many HBDs are oriented towards the lipid phase. The HBDs in transporters most likely play a dual role; on the one hand, they extract compounds with appropriate HBAs (i.e., allocrites) from the lipid membrane, and on the other, they allow allocrite gliding across the membrane[67]. In this context, phenyl residues that can undergo π-π stacking interactions with unsaturated rings in allocrites may also play a role (see Supplementary Figure 2A-D). The homodimeric Sav1866 from *Staphylococcus aureus*[18] is the prototypical type IV fold protein and a homolog of the monomeric Abcb1a and ABCB1. These proteins share the cross-over of helices: in the case of the homodimeric Sav1866, helices 4 and 5 from each monomer cross over to the other monomer; in the case of ABCB1, helices 4 and 5 from TMD1 cross over to the C-terminal TMD2 and helices 10 and 11 from TMD2 cross over to the N-terminal TMD1[6]. Because the X-ray structure of Sav1866 was obtained at a high resolution (3.0 Å)[18], it provides an ideal basis for modeling other type IV fold proteins.

Transporters with allocrites bound - structural insights

In recent years, several atomic structures of ABC transporters with allocrites bound have been resolved. Most of them are in the apo form. The first crystal structure of ABCB1 (at 4.5-3.8 Å resolution) was obtained with the hydrophobic cyclic peptides QZ59-RRR and QZ59-SSS bound to the transmembrane domain. The peptides and the transporter were assumed to connect via "hydrophobic" interactions[19]. Because the peptides carry several weak type I patterns formed by nitrogen and selenium, hydrogen bonding with the transporter is possible. The yeast mitochondrial ABC transporter Atm1 (an ortholog of human ABCB7) was crystalized (at 3.06 Å and 3.38 Å resolution) with the substrate glutathione. It revealed hydrogen bond formation between allocrite and transporter[70]. Locher and colleagues provided several atomic structures (at 4.0-3.2 Å resolution) obtained by cryo-electron microscopy (EM) of human ABCB1 with the three inhibitors elacridar, tariquidar, and zosuquidar, or vincristine bound[71]. The allocrites are surrounded by ample amino acids with π-electron systems or hydrogen-bonding groups (see, e.g., vincristine[71] or taxol[72]). The above inhibitors appear as pairs, arranged either in sequence (one behind the other) or in parallel or antiparallel orientation, respectively (see Ref.[71], Supplementary Figure 8 therein). Similar observations were made with two molecules of encequidar bound to ABCB1[73].

Similar to lipid membranes, transmembrane proteins are highly flexible and cannot be crystalized, unless they are stabilized. Molecules that may be favorable in this respect are dodecylmaltoside (DDM) that is an inhibitory detergent, inhibitory allocrites (see below), or the conformation-sensitive antibody UIC2 mAb.

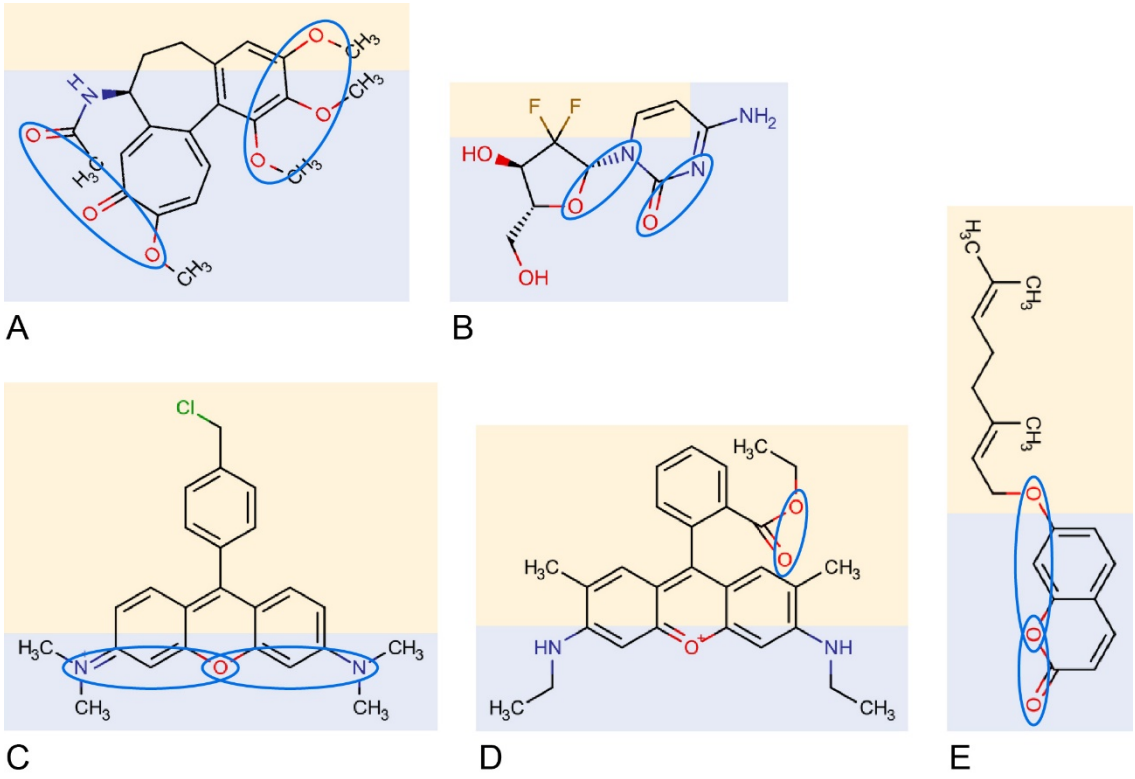

Figure 1. Examples of allocrites. Allocrites for ABCB1 are amphiphilic (polar part in blue, hydrophobic part in yellow) and carry type I or type II π-electron donor patterns (i.e., hydrogen bond acceptor patterns, HBAs) that are attracted by the HBDs in the protein. A type I pattern contains two HBAs separated by 2.5 ± 0.3 Å and a type II pattern contains two or three HBAs, where the outer two are separated by 4.5 ± 0.6 Å. Possible type I and type II patterns are encircled in blue: (A) *Colchicine*, two type II patterns. (B) *Gemcitabine* is an anticancer drug, which induces cell death by blocking DNA replication, with either two type I patterns (shown) or one type II pattern (not shown). (C) *Tetramethylrosamine* (*TMR*), type II patterns. (D) *Rhodamine 6G* (*R6G*), one type I pattern (secondary and primary amino groups are not involved in patterns). (E) *Auraptene*, a citrus phytochemical, one type I or one type II pattern. The orientation of the π-electrons in a pattern does not seem crucial. Unsaturated rings play a role in π-π stacking interactions.

The variation of crystal contacts under different crystallization conditions may also play a role in the wide distribution of conformations observed in ABC transporters[18,74,75]. Even if atomic structures may not reflect the functionally relevant conformations, they provide relevant aspects, such as the long predicted (i) broad binding areas that can accommodate two allocrites simultaneously ([76,77] see below); and (ii) the allocrite binding mode via weak dipolar interactions[64,65].

ALLOCRITE BINDING TO ABCB1 - A TWO-STEP PROCESS

Allocrite binding from water to the transporter characterized by the transporter-water binding constant K_{tw} occurs in two steps. The first step is allocrite partitioning from the aqueous phase into the lipid membrane, characterized by the lipid-water partition coefficient, K_{lw} (M^{-1}). The second step is allocrite attraction to the transporter within the membrane, characterized by the transporter-lipid binding constant K_{tl} (dimensionless). The transporter-water binding constant K_{tw} (M^{-1}) can therefore be expressed as the product of the partition coefficient K_{lw} and the binding constant K_{tl} within the membrane [Supplementary Equation 1].

For simplification, we use free energies of binding in the following instead of binding constants. They can be interpreted as affinities and are additive [Supplementary Equations 2-5]. The free energy of allocrite

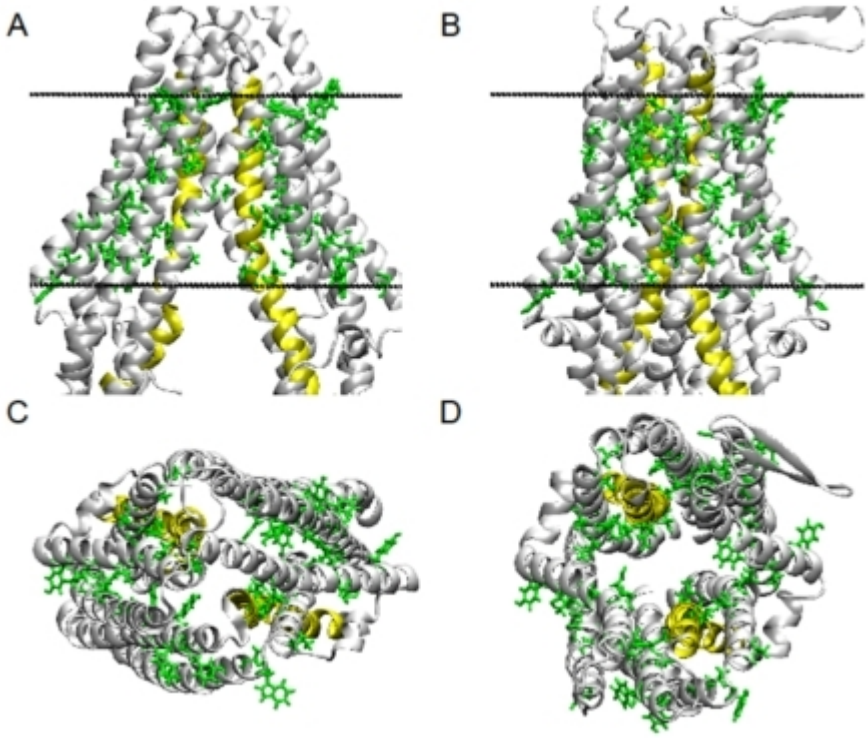

Figure 2. Amino acid residues with HBD side chains of ABCB1 at the level of the membrane (NBDs are truncated). (A) Abcb1a structure of the apo conformation open to the cytosol (PDB entry 4m1m) (side view). (B) Model of the closed conformation, based on the crystal structure of Sav1866 with two nucleotides bound (PDB entry 2hyd) (side view). (C) Apo conformation (top view). (D) Model of the closed conformation (top view). Amino acid side chains with HBDs are shown. TMD6 and TMD12 are colored yellow, whereas other helices are colored light gray. The two dashed lines indicate the position of the membrane (adapted from Ref.[67], for details see Supplementary Figure 1A and B).

binding from water to the transporter, ΔG^0_w, is the sum of the free energy of partitioning into the membrane ΔG^0_m (step I) and the free energy of binding to the transporter within the membrane ΔG^0_i (step II) [i.e., $\Delta G^0_w = \Delta G^0_m + \Delta G^0_i$, (Supplementary Equation 2)]. Methods for assessing the binding constants and the free energies of binding are detailed in the Supplementary Materials.

Quantification of the individual binding steps $\Delta G^0_{w(1)}$, ΔG^0_m, and $\Delta G^0_{i(1)}$

The free energy of binding from water to the activating binding region of the transporter in inside-out plasma membrane vesicles[78] and live NIH-MDR1-G185 cells was determined as $\Delta G^0_{w(1)}$ = -30 to -54 kJ mol^{-1}, the free energy of allocrite partitioning from water into the lipid membrane as ΔG^0_m = -23 to -34 kJ mol^{-1}, and the free energy of allocrite binding from the lipid membrane to the activating binding region of the transporter as the difference between these two free energies [Supplementary Equation 2], $\Delta G^0_{i(1)}$ = -7 to -27 kJ mol^{-1}. The values given refer to the lowest and highest values for each type of free energy of binding, among the 19 compounds investigated. ΔG^0_m contributes significantly to the overall binding, but the free energy of allocrite binding to the transporter within the membrane $\Delta G^0_{i(1)}$ varies more strongly than the free energy of lipid-water partitioning ΔG^0_m.

Characterization of the driving forces

The partitioning of uncharged amphiphiles such as drugs and detergents into electrically neutral membranes is driven by hydrophobic interactions. The classical hydrophobic effect (i.e., the release of ordered water molecules surrounding the solute in the aqueous phase upon partitioning into the membrane) is essentially entropy-driven (increasing with cooling), as observed for cyclosporine A[48] and nonionic detergents[79], for example. Partitioning of electrically charged drugs into an electrically neutral membrane is best described by the "non-classical hydrophobic effect", which is essentially enthalpy-driven (increasing with warming)[80]. The same is true for the cationic n-alkyl trimethyl ammonium chlorides (C_m-TACs) (see Ref.[79], Table 1 therein). Partitioning of amphiphiles decreases exponentially with increasing lateral packing density of the membrane, π_M, and increasing cross-sectional area of the partitioning amphiphile, A_D (Supplementary Equations 6 without and 7 and 8 with a negative surface potential, Ψ_m).

To extract amphiphiles out of the most hydrophobic environment in a cell, that is, the lipid bilayer, hydrophobic interactions are not useful. Amphiphiles such as drugs and detergents can however be extracted by dipolar interactions[81,82] [Figure 3]. An interesting aspect of the nature of dipolar interactions was revealed from a comparison of studies on allocrite binding to ABCB1 in liquid-crystalline and gel-state membranes[83,84]. Whereas partitioning of allocrites into the gel-state membrane was lower than into the liquid-crystalline membrane, as expected, because of the enhanced lateral packing density in the gel-state membrane [Supplementary Equation 6[58]], binding to the transporter within the gel-state membrane increased two to fourfold[84]. The two to fourfold increase in the binding constant to the transporter K_{tl} within the gel-phase membrane is consistent with a slight decrease of the dielectric constant ε_m in the gel phase as compared with that in the liquid-crystalline phase (see e.g., Ref.[61]) [Supplementary Equations 9-11].

Thus, partitioning into the membrane is driven by hydrophobic interactions and limited by the lateral membrane packing density π_m, whereas allocrite binding to the transporter within the membrane is driven exclusively by dipolar interactions that increase with increasing membrane packing density π_m and concomitantly decreasing dielectric constant ε_m.

ATP HYDROLYSIS IN INSIDE-OUT PLASMA MEMBRANE VESICLES

Early titrations of ABCB1 with drugs yielded bell-shaped activity curves

Monitoring ATP hydrolysis as a function of drug concentration was, and still is, the key experiment for understanding how ABC transporters catalyze allocrite transport or flopping. The first titrations of ABCB1 with drugs were performed with inside-out plasma membrane vesicles. As they expose the NBDs to the extravesicular aqueous phase, the release of inorganic phosphate during ATP hydrolysis can be easily monitored by spectroscopic techniques (see e.g.,[76]). The plasma membranes used originated from various ABCB1-overexpressing cell lines, including mouse embryo fibroblasts[85], ovarian carcinoma cells (2780^{AD})[86], insect cells[87,88], Chinese hamster ovary cells, CHRC5[89], CR1R12[90], CH^rB30 cells[91], and Ehrlich ascites tumor cells[76]. Most early ABCB1 titrations with drugs show a rise in steady-state ATPase hydrolysis at low concentrations up to a maximum, followed by a decrease at high concentrations, yielding characteristic *bell-shaped* activity (or velocity, V) *vs.* concentration curves [Figure 4]. ABCB1 titrations in plasma membrane vesicles with verapamil exhibited maximum steady-state ATP hydrolysis rates (V_{max}) around the concentration $C_{verap} \approx 10$ μM, even though the membranes originated from different cell lines. This may be due to the subtle compensation between the reduced allocrite partitioning into membranes of higher lateral packing density and the enhanced dipolar affinity between the allocrite and the transporter within the membrane. The drug-stimulated ATPase activity was directly proportional to the amount of P-glycoprotein, as demonstrated in Ehrlich ascites tumor cell lines[76], whereas the concentration of half-

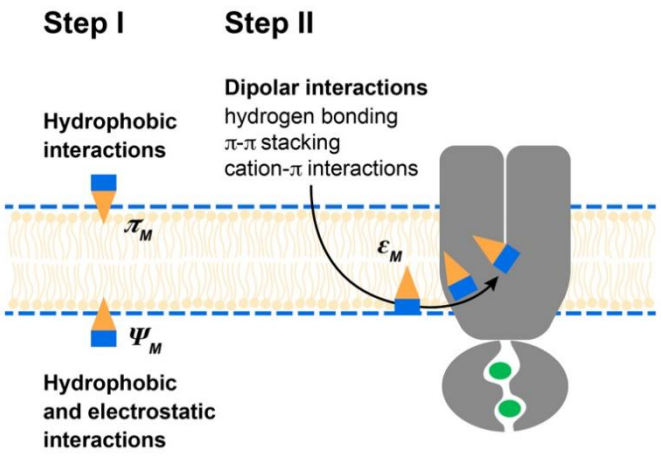

Figure 3. Scheme showing the types of interactions in the two-step process of allocrite binding from the aqueous phase to ABCB1 within the membrane. Step I: Partitioning of an amphiphilic allocrite (with polar part in blue and hydrophobic part in yellow) into the extracellular membrane leaflet depends on the lateral packing density of the membrane, π_m. Moreover, partitioning into the cytosolic leaflet depends in addition on the surface potential Ψ_m of the membrane[59]. Step II: Dipolar interactions between allocrite and transporter (including hydrogen bonding, π-π stacking, and π-cation interactions, given in the order of assumed relevance) are suggested to drive recognition, binding, and "transport" of the polar part to the middle of the membrane. Thereby it is assumed that the hydrophobic part remains in contact with the lipid environment.

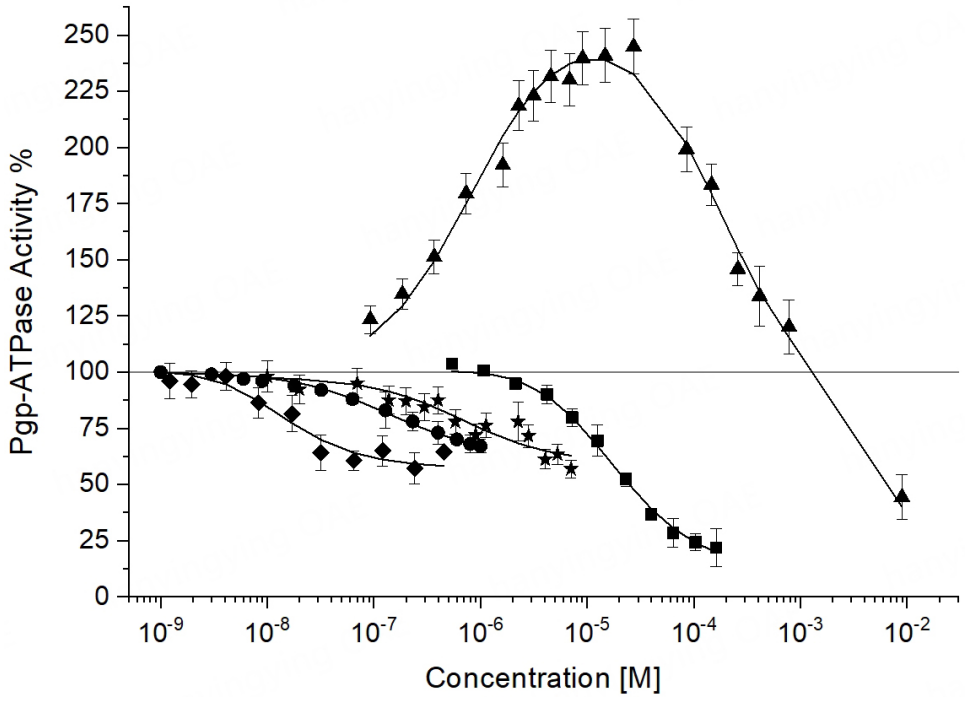

Figure 4. ABCB1 ATPase activity *vs.* allocrite concentration curves. ATPase activity measured in inside-out plasma membrane vesicles from NIH-MDR1-G185 cells[25]. Data are fitted to the two-site binding model by Litman *et al.*[96]. Tariquidar (diamonds); OC144 093 (circles), cyclosporine A (stars), DDM (squares), verapamil (triangles). Tariquidar, OC144 093, and verapamil inhibit as dimers. DDM[79] and cyclosporine A (see Ref.[79], Supplementary Table 3 therein) most likely inhibit as monomers due to their unfavorable $q = a_2/a_1$ values (see below).

maximum activation remained approximately constant. In reconstituted proteoliposomes, the

concentration of maximum activity depends on the type and residual concentration of detergents used for reconstitution, as discussed below.

Bell-shaped ATPase activity curves - artifact or fact?

Around the turn of the millennium, some skepticism arose regarding the inhibitory branch of ABCB1 activity curves and various artifacts were suspected of having influenced the titration curves at high drug concentrations, including membrane disordering, vesicle aggregation, ATP depletion, and a disturbed boundary layer around the protein. To clarify the ABCB1 kinetics, we investigated the potential artifacts.

Membrane disordering?

In 1995, Drori *et al.* observed that the cytotoxicity of anticancer drugs in multidrug-resistant cell lines was potentiated by chemosensitizers (i.e., detergents)[92]. They hypothesized that chemosensitizer-mediated membrane perturbations could interfere with the ATPase activity of ABCB1 and/or with ABCB1 drug transport capability. We know today that the detergents used by Drori *et al.* are allocrites for ABCB1 and act as ABCB1 inhibitors at higher concentrations[69,79,81,82,92].

To quantify the disordering effect of detergents and drugs at concentrations relevant for ABCB1 activation and inhibition, we measured the order parameter S_{mol} by D-NMR spectroscopy. The effects were negligibly small at concentrations of half-maximum activation, K_1, and showed a reduction in the order parameter of only about 5% in the case of detergents at half-maximum inhibition, K_2[79]. Somewhat more disordering was observed in the case of verapamil, owing to its larger cross-sectional area in the folded, membrane-bound conformation[93] (for quantitative data, see Supplementary Table 1 and references therein). Thus, ABCB1 eliminates allocrites before membrane disordering effects become obvious.

Moreover, a transition state analysis of ABCB1 activity in membrane environments exhibiting different lateral packing densities revealed that the transporter acts in a broad range of environments from densely packed lipids to loosely packed micelles (see Ref.[94], Figure 6 therein). With decreasing membrane packing density, the activation energy decreased and thus the rate of ATP hydrolysis increased. Whereas the activity was entropy-driven at a high packing density, it was essentially enthalpy-driven at a low packing density[94]. The activity of ABCB1 is thus robust with respect to its environment and it works perfectly in a much broader range of membrane packing densities than encountered under physiological conditions.

Vesicle aggregation?

In inside-out plasma membrane vesicles, the negatively charged inner membrane surface is exposed and thus these vesicles repel each other. Upon titration with strongly cationic drugs, the negative surface potential is strongly reduced, which may lead to vesicle aggregation at high concentrations. Even under these conditions, a decrease in activity occurs before aggregation takes place. The phenomenon of vesicle aggregation at high drug concentrations is limited to compounds with $pK_a > 9$. Cationic drugs with lower charge or no charge exhibit no vesicle aggregation, and show bell-shaped curves as well (for details, see[78]).

ATP depletion?

ATP depletion has been observed with pluronic block copolymers[95] at an incubation time of 2 h. This is at least twice the incubation time generally used in ATPase activity measurements. The titration of ABCB1 with verapamil under ATP regenerating conditions again yielded practically identical bell-shaped activity *vs.* concentration curves (see Ref.[66], Figure 2 therein).

Disturbed boundary lipids?

As mentioned above, boundary lipids or lipid domains were not observed in natural membranes at physiological temperatures upon monitoring different deuterated phospholipids by D-NMR spectroscopy[50]. Disturbed boundary layers, that is, disordered membranes of lower packing density, would enhance rather than reduce the steady-state ATP hydrolysis rate (for details, see below). We conclude that the decrease in ABCB1 ATPase activity at high drug concentrations is real and highly relevant for understanding transporter inhibition (see below).

Two-site binding models are required

In 1997, Stein and colleagues proposed a model for quantitative evaluation of bell-shaped ABCB1 activity *vs.* allocrite concentration curves[76,96]. This model is based on the principle of Michaelis-Menten kinetics and takes into account basal activity, V_0, in the absence of an exogenous allocrite; enhanced activity, V_1, with one allocrite molecule bound to the transporter; and reduced activity, V_2, with two allocrite molecules bound to the transporter [Supplementary Equation 12] (see Figure 4). A related model, however, assuming basal activity, uncoupled from transport, a drug-activated phase, and a drug-inhibited phase, was later proposed by Al-Shawi and colleagues[77]. The recent advances in atomic transporter structures provide direct evidence for *two* molecules bound to ABCB1 (see e.g., Ref.[71]) and support the necessity of two-site binding models. We consider the model proposed by Stein and colleagues[76,96] as more plausible because empty cycling seems unlikely from an energetic point of view.

Allocrites that may contribute to basal activity in plasma membrane vesicles

Basal activity was proposed to be due to an as-yet-unknown allocrite[10]. Different endogenous allocrites may be considered. The most prevalent among them is POPC. Protonated POPC ($POPC^+$) shows the typical characteristics of an ABCB1 allocrite and may be responsible for basal activity. Despite the low intrinsic pK_a value of the phosphate group in the pure phosphatidylcholine (PC) monolayers[97], a small fraction of PC molecules may be protonated at the phosphate group in the overall negatively charged cytosolic membrane leaflet of cells. As the flipping rate of PC lipids (from the extracellular to intracellular membrane) is low[98], and ABCB1 may cope approximately by flopping them back to the extracellular membrane leaflet, the concentration of $POPC^+$ in the cytosolic membrane leaflet remains low and prevents inhibition of the transporter. ABCB1 may thus contribute to the maintenance of lipid asymmetry in biological membranes, as suggested earlier[63,99]. The particularly low basal activity in DPPC vesicles[27] could be due to a very low concentration of the cationic species in the absence of negatively charged lipids, or if the zwitterionic form is also an allocrite, to the an excessive and thus inhibitory concentration of DPPC. Thus, basal ABCB1 activity may arise from flopping $POPC^+$, or POPC in general.

The factors influencing the steady-state ATP hydrolysis rate

Correlation between the steady-state ATP hydrolysis rate and allocrite affinity $a_{\phi_{11}}$

Stein and colleagues studied the correlation between kinetic parameters and the lipid-water partition coefficient as well as the van der Waals surface area of drugs in inside-out plasma membrane vesicles of CR1R12 cells[96]. With the exception of valinomycin, a good correlation between the surface area of drugs and the compound's affinity to ABCB1 was observed. Similar data were provided by Sharom and colleagues[99].

A good correlation between size-related parameters and the compound's affinity to the transporter within the lipid phase $a_{\phi_{11}}$ can be rationalized by assuming that increasing the surface area of the drug requires increasing the number of hydrogen-bonding groups to prevent aggregation. Therefore, increasing the molecular surface area (or molecular weight) roughly correlates with increasing the number of HBAs in

ABCB1 (= O, -O-, -N <), which can interact with HBDs in the transporter. If one considers that valinomycin offers a number of π-electron donor groups for complex formation with potassium, it is no longer an outlier[96]. The correlation between HBAs and the molecular weight of allocrites is also displayed in Supplementary Figure 3. Thus, the observed decrease in activity with increasing affinity can be explained by weak dipolar interactions between the available HBAs in allocrites and the HBDs in the protein.

A reduced allocrite affinity with a concomitant increase in activity was also obtained by eliminating "anchor points" in the protein binding region, for example, by mutating amino acids able to form π-π stacking or hydrogen bonding interactions with allocrites. Compounds that were "inhibitors" in the native transporter became activators in the mutant[100,101], supporting the inverse correlation between affinity $\Delta G^0_{(1)}$ and the steady-state ATP hydrolysis rate. Thus, the steady-state ATP hydrolysis rate and transport decrease with an increasing number of weak dipolar interactions (see e.g., TMR and R6G in Figure 1 from Ref.[102]). Using a broader range of tetramethylrosamine (TMR) analogs and their xanthone precursors, Tombline and colleagues[102] demonstrated that, in addition to the number of HBAs, the logP (octanol-water partition coefficient, used as a crude estimation of lipid-water partition coefficient) plays an additional role (e.g., Chart 2, compounds 14-16 in Ref.[102]). A strict correlation between the rate and $\Delta G^0_{(1)}$ holds true only if ΔG^0_{el} is rather constant. More diverse sets of compounds require the inclusion of ΔG^0_{el} (see below).

The steady-state ATP hydrolysis rate (lnV_1) decreases linearly with $\Delta G^0_{(1)}$

An approximately linear decrease of the steady-state ATP hydrolysis rate, lnV_1 (on a logarithmic scale), with decreasing free energy of binding $\Delta G^0_{(1)}$ (or increasing allocrite affinity to the transporter) was observed with various data sets[78,79,82]. Figure 5A shows lnV_1 *vs.* $\Delta G^0_{(1)}$ for very diverse types of allocrites, including moderately charged (on the upper diagonal line), highly charged (on the lower diagonal line), and essentially uncharged amphiphiles (between the two diagonal lines). Figure 5A, moreover, includes molecules of low amphiphilicity, such as PSC 833, cyclosporine A, OC144-093, and tariquidar, or molecules of unfavorable amphiphilicity, such as DDM[79] (below the lower diagonal line). The strict linear dependence of lnV_1 *vs.* $\Delta G^0_{(1)}$ thus exists only within a specific charge group. To understand this phenomenon, we assessed the free energy contribution per single hydrogen bond, ΔG^0_{HBA}, and per single charge, ΔG^0_{el}.

ΔG^0_{HBA} was assessed by dividing ΔG^0_i by the number of HBAs in type I and type II patterns of the different compounds (Figure 5B, y-axis). These values were plotted as a function of the number of HBAs per compound (Figure 5B, x-axis). A higher number of HBAs per compound was associated with a lower contribution per single HBA, suggesting that not all HBAs present contribute simultaneously to binding in the case of larger compounds with a higher number of HBAs. The value per single hydrogen bond was estimated by extrapolation [Figure 5B]. The values given in Figure 5B were evaluated for mouse embryo fibroblast membranes.

The free energy of binding per charge and HBA - comparison with literature data

To assess the affinity of a full cationic charge to ABCB1, we used the quaternary ammonium ion in C_m-TACs. The free energy of binding per cationic charge to ABCB1 was assessed as ΔG^0_{el} = -6.5 ± 0.7 kJ mol^{-1}[79] (see Figure 5B). The value is within the range of π-cation interactions[103].

For electrically neutral detergents with a very peripheral location of the polar head group, and progesterone, which lacks a strong type I pattern, extrapolation to a single HBA yields $\Delta G^0_{HBA} \approx$ -3.5 kJ mol^{-1} [Figure 5B]. For

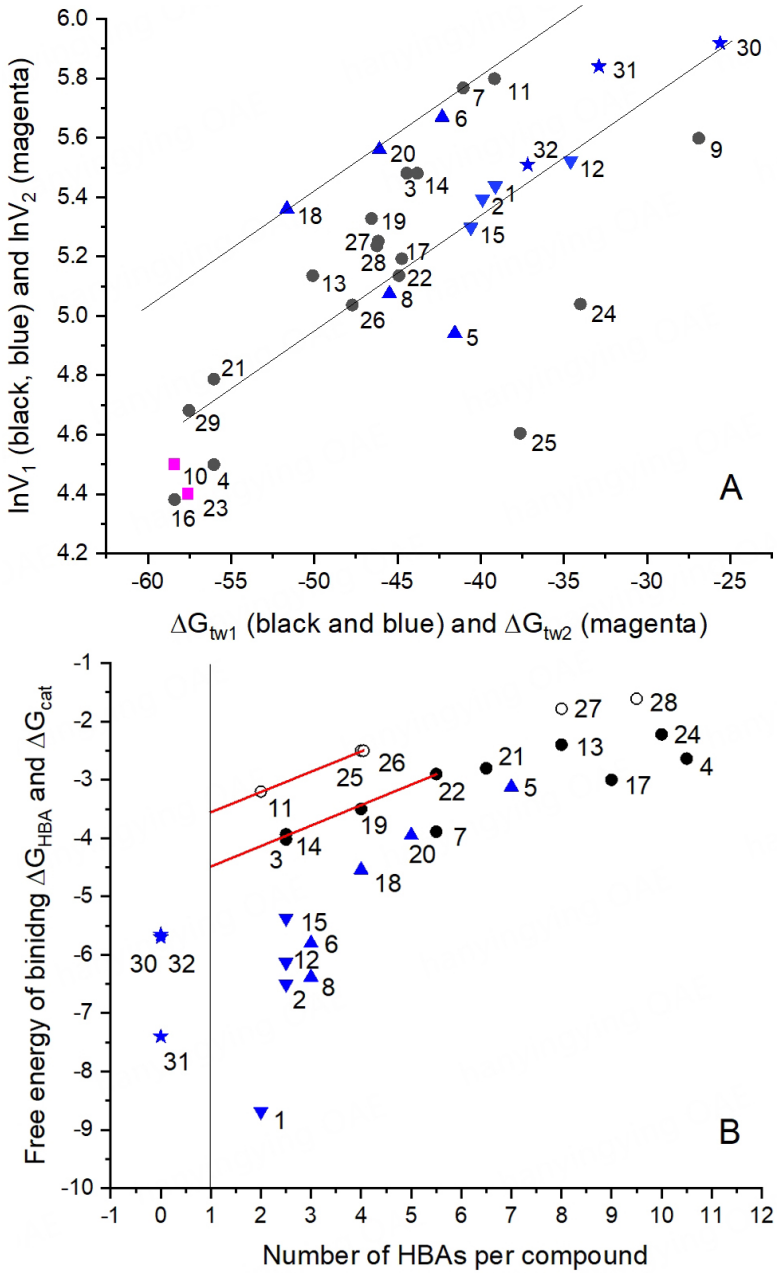

Figure 5. Correlations between maximum steady-state ATP hydrolysis rate and affinity in mouse embryo fibroblast membranes. (A): ln(V_1) *vs.* the free energy of binding, ΔG_{tw}. The maximum steady-state ATP hydrolysis rate, V_1, is expressed as a percentage of the basal steady-state ATP hydrolysis rate taken as 100%. Data were obtained from phosphate release measurements: (1) amitriptyline, (2) chlorpromazine, (3) cis-flupenthixol, (4) cyclosporine A, (5) daunorubicin, (6) dibucaine, (7) diltiazem, (8) glivec, (9) lidocaine, (10) OC144-093, (11) progesterone, (12) promazine, (13) reserpine, (14) trifluoperazine, (15) trifluopromazine, (16) PSC 833, (17) vinblastine (1-17, from Ref.[78]), (18) amlodipine, (19) nimodipine, (20) verapamil (18-20, from Ref. [93]), (21) sirolimus, (22) tacrolimus (21-22 from Simon Lang and A.S., unpublished results), (23) tariquidar (X. Li-Blatter and A.S., unpublished results), (24) etoposide (from Ref.[66]), (25) C_{12}-maltoside, (26) C_{13}-maltoside, (27) $C_{12}EO_8$, (28) Triton X-100, (29) Tween 80, (30) C_{10}-TAC, (31) C_{12}-TAC, (32) C_{14}-TAC (25-32, from Ref.[79]). Black filled circles: neutral compounds or compounds exhibiting low charge ($pK_a \leq 8$). Blue upward-pointing triangles: cationic compounds with intermediate charge ($pK_a \geq 8$). Blue downward-pointing triangles: strongly charged cationic compounds ($pK_a \geq 9$). (B): The free energy of binding per single cationic full charge, ΔG_{tw}, and single HBA, as a function of the number of patterns per compound n_{HBA}. Compound numbers and symbols as in (A), black open circles, uncharged compounds. The free energy per hydrogen bond (y-axis) decreases with the increasing number of hydrogen bonds in patterns per compound (x-axis). We extrapolated to one hydrogen bond in the case of electrically neutral compounds and compounds with low charge (red lines).

electrically neutral drugs likely penetrating more deeply into the membrane, extrapolation to a single hydrogen bond yields $\Delta G^{0}_{HBA} \approx$ -4.5 kJ mol^{-1}. Highly charged compounds such as amitriptyline (pK_a 9.4) exhibit more negative free energy of binding, ΔG^{0}_{HBA} = -8.7 kJ mol^{-1} [Figure 5B], owing to a charge contribution. Because most cationic drugs show lower pK_a values than amitriptyline, charge contributions are generally low and hydrogen bonding dominates.

The calculated free energy of hydrogen bond formation is on the order of -20 kJ/mol (see e.g.,[104]) and is thus much higher than the free energy per HBA assessed for ABCB1. However, our values agree well with the rare *measured* free energies of hydrogen bond formation in large membrane proteins. The H-bond strength of a single Cα-H···O H-bond in the transmembrane helical dimer of glycophorin A, located in the center of the membrane, was assessed as 3.7 kJ mol^{-1}, using vibrational frequency shifts of dimeric and non-dimeric variants of glycophorin A, containing a deuterium-labeled Gly[105]. The average contribution of eight interhelical side-chain hydrogen-bonding interactions throughout bacteriorhodopsin, reconstituted in DMPC (1,2-dimyristoyl-sn-glycerol-3-phosphocholine)/CHAPSO {3[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane-sulfonate}, was determined as 2.5 kJ mol^{-1}[106]. The sign of free energy of binding depends on the reference state.

The role of amphiphilicity for the steady-state ATPase rate

Amphiphilicity is a qualitative term used to describe a molecule exhibiting a polar part and a non-polar, hydrophobic part. As shown previously, the steady-state ATP hydrolysis rate of detergents and drugs strongly depends on ratio $q = \Delta G^{0}_{A}/\Delta G^{0}_{T}$ (ratio of the free energy of allocrite binding to the hydrophobic membrane and to the more polar transporter)[79].

The ratio q can be considered as a quantitative description of a compound's amphiphilicity. For ATPase-activating compounds such as the cationic C_m-TACs, the ratio is $q \approx$ 3-4, for cationic drugs, $q \approx 3$, and for electrically neutral detergents, $q \approx 2.7$. Electrically neutral detergents are particularly sensitive to the ratio q. Slight deviations to higher values (e.g., C_{14}-maltoside with $q \approx 3$) lead to a lack of activation/inhibition because the affinity to the membrane is too high. Lower values lead to inhibition [e.g., C_{12}-maltoside (DDM) with $q = 2.5$]. Inhibitory drugs including cyclosporine A show low ratios ($q \approx 1$) (see Supplementary Information to Ref.[79]).

Conclusions on the mechanism of transport

Altogether, we demonstrated that the steady-state ATPase hydrolysis rate decreases with increasing affinity of the allocrite from water to the transporter $\Delta G^{0}_{A/T}$. However, the rate is particularly sensitive to charge and amphiphilicity. Compounds with two HBAs and a very weak cationic charge or only two HBAs, providing a free energy of binding comparable to that of a single cationic point charge of ΔG^{0}_{ion} = -6.5 ± 0.7 kJ mol^{-1}, induce a higher ATP hydrolysis rate than the latter. Weak dipolar interactions are thus more favorable for transport than a single strong cation-π interaction. Weak interactions thus may allow gliding of the polar part of the allocrite across the transporter binding region towards the center of the transporter in the middle of the membrane (which exhibits the lowest dielectric constant and thus the highest affinity to the transporter) and may facilitate flopping. While the polar part of allocrites forms weak electrostatic interactions with the transporter, the hydrophobic part seems to remain associated with the lipid membrane, as proposed earlier[81]. The slow-down in the steady-state ATP hydrolysis rate with reduced amphiphilicity is consistent with such an interfacial flopping process. An interfacial transport process has been described for the oligosaccharide transporter PglK with two ATPγS molecules bound[107].

ATP HYDROLYSIS IN RECONSTITUTED PROTEOLIPOSOMES AND DETERGENT

MICELLES

Proteoliposomes with residual detergents show higher *Km (K1)* and lower *Vmax (V1)* values

The concentrations of half-maximum activation K_m (Michaelis-Menten constant) or K_1 [Supplementary Equation 12] of specific allocrites in proteoliposomes are often distinctly higher than in natural plasma membrane vesicles. If ABCB1 is reconstituted into lipid vesicles, detergents are used. Even though such detergents are usually removed after reconstitution, residual detergents may remain. Reconstitution of ABCB1 by solubilization in octyl glucoside (OG) in the presence of *Escherichia coli* lipids and subsequent titration with verapamil yielded an almost 10-fold higher concentration of maximum activation (e.g.,[108,109]). Still higher concentrations of maximum activation (e.g.,[71]) or even a loss of activity[91] were observed when DDM was used. Callaghan and colleagues prevented "inactivation" by a careful reconstitution protocol that included excess crude lipid mixtures and extensive gel filtration to eliminate DDM[91]. The often-observed shift of K_m values to higher concentrations is caused by competitive inhibition of the allocrite (e.g., verapamil)[82]. An increase in K_m, combined with a decrease in V_{max}, is typical for competitive inhibition. The inhibitory power of detergents increases in the order CHAPS < OG < DDM[27] (OG and DDM[79], CHAPS: X.L. and A.S., unpublished results) [Figure 4].

The loss of activity of transporters reconstituted in lipid bilayers is thus caused by residual detergents such as OG and particularly DDM that act as competitive inhibitors. ATPase activity is regained by dilution or squeezing out (see, e.g., effects observed by addition of cholesterol in Ref.[110,111]).

Detergent micelles show higher *Km (K1)* and higher *Vmax (V1)* values

The basal ATPase activity of Sav1866, reconstituted in liposomes, increased two- to threefold upon the addition of detergents above their critical micelle concentration (CMC), which led to the formation of mixed micelles (see Ref.[32], Figure 5 therein).

Ambudkar and colleagues[112] compared the basal ATPase activity of mP-gp (the murine analog of ABCB1) in native High Five insect cell membranes (42 to 54 nmol P_i/min/mg of protein) and in DDM micelles (79 to 83 nmol P_i/min/mg of protein). In DDM micelles, the basal ATPase activity was again about twice as high as in insect cell membranes. Moreover, a 30-150-fold decrease in the apparent affinity for verapamil and cyclic peptide inhibitor QZ59-SSS was observed in detergent micelles compared with that in native or artificial membranes. Consequently, the cyclic peptide "inhibitor" QZ59-SSS and the modulators zosuquidar, tariquidar, and elacridar (inhibitors in lipid vesicles with IC_{50} values in the 10-40 nM range) stimulated the ATPase activity of purified human or mouse P-gp in DDM micelles.

Micelles exhibit higher dielectric constants, ε_m, than lipid vesicles, leading to a substantial decrease in allocrite affinity to the transporter (see Supplementary Equations 9-11) and a concomitant increase in ATPase activity and transport, as expected. Thus, ABCB1 remains functional in a micellar environment, although transport becomes Sisyphean because of the low lateral packing density, π_M, of micelles.

Cholesterol enhances the membrane packing density and allocrite affinity

The addition of cholesterol has often enhanced ABCB1 activity (see, e.g., effects observed by addition of cholesterol in Ref.[110,111]). The addition of lipids generally dilutes residual inhibitory detergents such as OG and DDM. Densely packed lipids, including cholesterol, may in addition squeeze detergents out of the lipid bilayer, which leads to enhanced ATPase activity.

However, the specific effect of cholesterol (in the absence of detergents) is to slightly reduce the steady-state ATP hydrolysis rate[84,94]. Cholesterol enhances the lateral membrane packing density, π_M, and reduces the

dielectric constant, ε_m, of a lipid bilayer (see above), with the consequence of an enhanced dipolar attraction of the allocrite to the transporter within the membrane.

These predictions are consistent with experiments by Ueda and colleagues[113], who measured the modulation of drug-stimulated ATPase activity of ABCB1 by cholesterol in the absence of OG or DDM. They reconstituted ABCB1 in membranes with different cholesterol contents (C_{ch} = 0%-20% w/w) and used ten allocrites with increasing molecular weights from 345 to 1111 Da (see Ref.[113], Table 1 therein). For small molecules (molecular mass < 500 Da), K_m decreased (i.e., the affinity to the transporter increased) by about a factor of two with increasing cholesterol content. For larger molecules, K_m remained approximately constant, and for the largest molecule, K_m even increased slightly with increasing cholesterol content. For small compounds, lipid-water partitioning is not limiting and the affinity to the transporter within the membrane increases with decreasing dielectric constant ε_m. Conversely, in the case of the largest compounds, the affinity to the transporter slightly decreased because partitioning into the membrane became the limiting factor. With increasing cholesterol content, the activity, V_{max}, clearly decreased for large molecules with many HBAs, whereas it remained approximately constant for small molecules. Although the effects are minor, the data[113] perfectly agree with the above expectations. Sharom and colleagues obtained related results and also demonstrated that "the cholesterol content of the membrane has only a modest influence on both the basal and the drug-stimulated ATPase activity of P-gp"[114].

The consequences of using methyl-β-cyclodextrin (mβCD) for cholesterol elimination

Elimination of cholesterol by mβCD often leads to a strong decrease in ABCB1 activity. On the basis of this observation, it was concluded that cholesterol must be strongly ABCB1-enhancing, but this is not the case (see above). Elimination of cholesterol with mβCD is complex. The cyclic polysaccharide has a central hydrophobic cavity that can be occupied by cholesterol, lipids, or any other hydrophobic or amphiphilic molecule. In its empty form, mβCD engulfs cholesterol from the hydrophilic end, thereby partially penetrating into the head group region of the lipid membrane. Sharom and colleagues[114] found a decrease in ABCB1 activity with increasing concentrations of mβCD in CH^RB30 plasma membrane vesicles, in DMPC proteoliposomes, and in CHAPS micelles, independent of cholesterol and suggested the possibility of a direct interaction between ABCB1 and mβCD[114]. mβCD carries multiple hydrogen bond acceptor patterns, and therefore direct interaction with ABCB1 is highly likely. It seems to start already at low concentrations (X.L-B and A. S. unpublished results). Because membranes disintegrate at higher mβCD concentrations, obtaining definitive experimental proof of this is difficult.

To inhibit endogenous cholesterol synthesis, lovastatin was used in addition to mβCD[115]. Lovastatin is also a modulator of ABCB1 directly inhibiting ABCB1 at the concentrations used (A.S. unpublished results). Thus, the strong effects observed upon cholesterol elimination with mβCD are likely caused by direct inhibition of ABCB1 by mβCD at low concentrations and by general delipidation at higher concentrations.

Thus, cholesterol elimination with mβCD strongly reduces ATPase activity, but cholesterol supplementation in a biological membrane would not enhance it.

ATP HYDROLYSIS IN LIVING CELLS

At this point, the altered "*membrane permeability*"[1] of cells in the presence of ABCB1 becomes relevant. Whereas in inside-out vesicles, active transport and passive diffusion work in the same direction, they work in opposite directions in cells. This phenomenon was described as the "pump-leak effect"[116]. Here, the balance is in favor of export (pump) in the case of large molecules, and in favor of influx (leak) in the case of smaller ones[58]. The comparison of ATPase activity measurements in inside-out plasma membrane vesicles

and cells is therefore of special interest.

Cells cultured in the presence of glucose generally work under glycolytic conditions[117]. Under these conditions, the extrusion of one lactate corresponds to one ATP synthesized. As ATP is synthesized according to requirements, ATP hydrolysis can be monitored by measuring the steady-state extracellular acidification rate (ECAR) using a Cytosensor microphysiometer[117,118]. The drug-stimulated ABCB1 activity was obtained by comparing MDR1-transfected mouse embryo fibroblasts (NIH-MDR1-G185 cells) with the corresponding wild-type cells (NIH-3T3 cells). If the energy requirement is enhanced, cells possess the ability to dynamically switch to oxidative phosphorylation (or respiration)[119]. To remain under glycolytic conditions and prevent potentially toxic side effects, drugs were applied for short time intervals of 2-3 min[59,120] and were washed out after each stimulation (see Supplementary Figure 4).

Small molecules: ABCB1 titration curves in cells and vesicles are similar

Small cationic drugs equilibrate rapidly between the inner and outer plasma membrane leaflets, and thus the concentrations of half-maximum activation K_i are similar in cells and inside-out vesicles [Figure 6A and D or B and E]. The slightly lower K_i values in cells are likely due to the somewhat lower cytosolic free magnesium ion concentration in cells and thus to a somewhat more negative membrane potential compared with that in vesicles[32,112]. Notably, the ATPase activity induced by small allocrites was more than twofold higher in cells than in plasma membrane vesicles of the same cells, even at the short stimulation times[69]. Significantly higher steady-state ATP hydrolysis rates in cells than in proteoliposomes [Figure 6C and F] were also measured for CFTR (ABCC7)[14].

Large molecules: titration curves in cells and vesicles differ, revealing transport

For slowly diffusing compounds, such as daunorubicin (MM: 527.5 Da), a known ABCB1 "substrate" [Figure 6B], the ATPase activity profiles of inside-out vesicles and cells differed distinctly. At identical aqueous concentrations (e.g., C_{dau} = 1 μM), daunorubicin inhibited ABCB1 activity in inside-out vesicles and activated it in living cells. The drug concentration in the cytosolic membrane leaflet of cells was estimated according to the new K_i value to be approximately one hundreds that in the cytosolic membrane leaflet of inside-out plasma membrane vesicles[69]. In the case of daunorubicin and other "substrates", ABCB1 can maintain a concentration gradient (between the inner and outer plasma membrane leaflets) or, in other words, can cope with influx. Transport, reflected by a substantially reduced K_i value in the cytosolic membrane leaflet of cells, was also observed for Tween (reduced by about one-thousandth)[69] and vinblastine (reduced by about one-tenth) (see Ref.[78], Figure 3A-E therein). Interestingly, even detergents such as Triton X-100 and $C_{12}EO_8$ showed a reduction to approximately one-tenth in the cytosolic membrane leaflet of cells[69].

ABCB1 efficiently competes with passive influx of large allocrites, that is "substrates" (see Ref.[35]). Therefore their concentration in the cytosolic plasma membrane leaflet of cells can be significantly lower than in the cytosolic leaflet of inside-out plasma membrane vesicles of the same cells. These measurements provide the first unequivocal insight into the correlation between ATPase activity and transport.

Stoichiometry: one ATP hydrolyzed per allocrite transported

The stoichiometry of ATP-driven ion transporters moving ions from the aqueous phase at one side of the membrane to the aqueous phase at the other side of the membrane has long been determined[121]. Assessing the stoichiometry of ATP-driven allocrite transport by ABCB1 has proven significantly more challenging. Allocrites are captured in the cytosolic membrane leaflet, and are flopped to the extracellular leaflet[122]. From the extracellular leaflet, they eventually partition into the aqueous phase according to their lipid-water partition coefficient. Thus, many drugs accumulate within membranes.

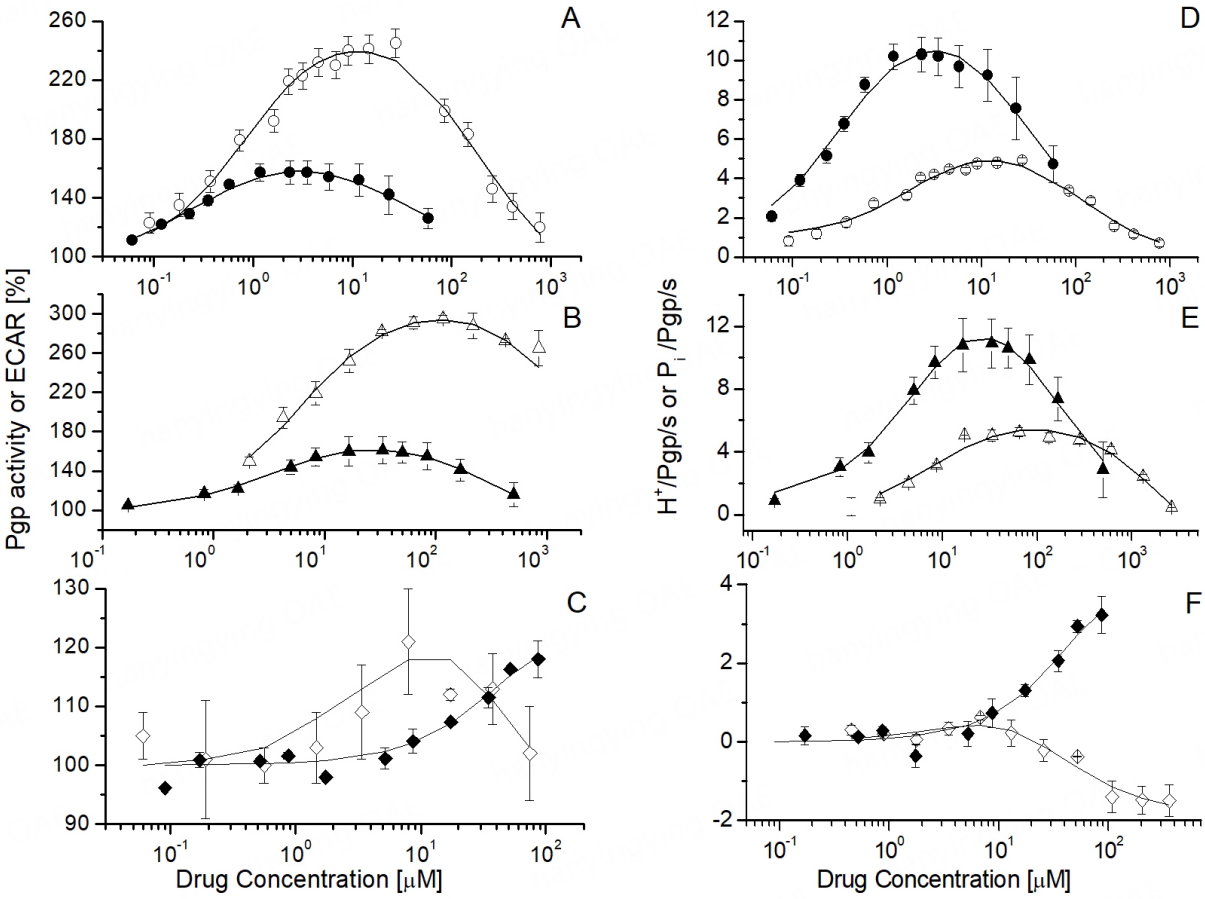

Figure 6. ABCB1 ATPase activity in NIH-MDR1-G185 cells (filled symbols) and inside-out plasma membrane vesicles (open symbols) induced by three drugs. (A-C) ATPase activity expressed as percent of the basal value in the absence of drugs. (D-F) ATPase activity expressed as protons (i.e., lactic acid) or phosphate released per ABCB1 per second. Calculations are based on the expression level of ABCB1 determined for NIH-MDR1-G185 cells [124]. (A and D) Verapamil (•); (B and E) diltiazem (▲), and (C and F) (♦) daunorubicin. Lines are fits to Supplementary Equation 12. Standard deviations are shown (taken from Ref.[69]). With copyright permission from BBA.

To circumvent the problems caused by allocrite partitioning into the membrane and passive diffusion across the membrane, Eytan *et al.* measured the ATP-dependent uptake of the $^{86}Rb^+$-valinomycine complex into proteoliposomes[123]. They suggested 1.2-2 ATPs hydrolyzed per transported $^{86}Rb^+$-valinomycine complex.

Stein and colleagues[124] used vinblastine, a known "substrate," as a test molecule. Vinblastine exhibits a comparatively low lipid-water partition coefficient, $K_{lw} \approx 15\ M^{-1}$ (in the absence of DMSO) [Table 1] (Li-Blatter X, unpublished results), a relatively large cross-sectional area (A_D = 140 Å^2), and accordingly relatively slow diffusion. Maximum outward pumping from NIH-MDR1-G185 mouse embryo fibroblasts loaded with vinblastine was assessed as 2.1×10^6 molecules s^{-1} $cell^{-1}$ with a turnover of 1.1 molecules s^{-1}. Vinblastine uptake in the absence and presence of the inhibitor verapamil (at inhibitory concentration, c = 50 μM) yielded maximum outward pumping of 2.73×10^6 molecules s^{-1} $cell^{-1}$. The turnover was 1.4 molecules s^{-1}[124]. Comparing these values with the steady-state ATP hydrolysis rate in inside-out plasma membrane vesicles (V_{max} = 3.5 ABCB1 $molecules^{-1}$ s^{-1}) suggested that about two ATPs hydrolyzed per vinblastine transported[124]. At the low concentrations used for data evaluation ($C_{vin} \leq 10$ μM) and the short stimulation times[124], toxicity was most likely negligible.

Table 1. ABCB1 allocrites used as inhibitors

Allocrites	MW	Log P[4)]	K_{lw}	$K_{tl(2)}$	$K_{tw(2)}$	ΔG^0_{tw}	$\Delta G^0_{tl(2)}$	$\Delta G^0_{tw(2)}$ 1)
	[Da]		[M^{-1}]	[M^{-1}]	[M^{-1}]	[kJmol^{-1}]	[kJmol^{-1}]	[kJmol^{-1}]
Cyclosporine A[48]	1202.6	2.9	5.2*10^3	1.3*10^2	6.7*10^5	-32.4	-12.5	-44.9
Taxol2)	*853.92*	~3						
Vinblastin2)[78]	*811.0*	*3.7*	*1.5*101*	*1.6*103*	*2.4*104*	*-17.3*	*-19.1*	*-36.4*
Encequidar[3)]	688.7	5.8	-	-	-	-	-	-
Tariquidar[3)][Li-Blatter X, unpbl.]	646.7	6.1	3.8*10^4	2.4*10^3	9.1*10^7	-37.5	-20.1	-57.6
Elacridar[3)]	563.6	5.6	-	-	-	-	-	-
Zosuquidar[3)]	527.6	4.9	-	-	-	-	-	-
OC144-093[3)][78]	494.7	7.3	-	-	1.1*10^6	-	-	-46.1
Verapamil[93]	454.6	3.8	4.7*10^2	5.810^1	2.7*10^4	-26.2	-10.4	-36.6

[1)]All allocrites listed inhibit as dimers, except cyclosporine A, which may inhibit as a monomer. The free energy of binding of the second, inhibitory molecule $G_{tw(2)}$ is always less negative than the free energy of binding of the first, activating molecule $\Delta G_{tw(1)}$[59]. [2)]Taxol and vinblastine are not useful as inhibitors, taxol because of its low solubility[125] and vinblastine because of its relatively low affinity to the membrane (see Table 1). [3)]The high affinity of encequidar, tariquidar, elacridar, zosuquidar, and OC144-093 is not due to a particularly high affinity to the transporter, but to a particularly high. [4)]LogP values are from https://pubchem.ncbi.nlm.nih.gov

We monitored ATP hydrolysis in the same NIH-MDR-G185 cells and the corresponding wild-type cells as a function of vinblastine concentration using a Cytosensor microphysiometer[78]. The concentration of half-maximum activation of vinblastine, K_1, in live cells shifted to an approximately 10-fold higher concentration than that in the inside-out plasma membrane vesicles of the same cells. Although the steady-state ATPase rate was higher in cells than in plasma membrane vesicles, the turnover number at $C_{vin} \leq 10$ μM was only about 1.4 s^{-1} because of the K_1 shift from 1.6 μM in inside-out vesicles to about 16 μM in cells. The kinetic data obtained in live cells support a one-to-one stoichiometry. Using rhodamine 123 as a "substrate," Shapiro and Ling[126] also proposed a one-to-one stoichiometry. Further arguments supporting a one-to-one stoichiometry are discussed elsewhere[33]. Under inhibitory conditions, where two allocrites are bound, the hydrolysis of one ATP most likely allows flopping of two allocrites, although at a low rate.

ABCB1 INHIBITION

A broad binding region allows for allosteric or competitive inhibition

In principle, any allocrite that can reach the inhibitory phase of a bell-shaped ATPase activity titration curve can act as an inhibitor (see, e.g., verapamil in Figure 4), provided it is soluble at the concentrations required. Inhibition is moreover obtained by compound combinations. Stein and colleagues[127] assessed three categories of interactions in the drug binding region of ABCB1: (i) cooperative stimulation between verapamil and amphiphilic molecules smaller in size than verapamil (e.g., progesterone); (ii) allosteric inhibition between verapamil and molecules of similar size (e.g., daunorubicin); and (iii) competitive inhibition between verapamil and molecules larger in size such as cyclosporine A (see Supplementary Figure 3). Competitive inhibition was moreover observed between vinblastine, verapamil, cyclosporine A, and lipids[99] or between verapamil, cyclosporine A, and the detergents Triton X-100, $C_{12}EO_8$, and Tween 80[82]. Depending on the concentration applied, detergents such as polyethylene glycol and Tween that are often used as excipients in drug formulations (see, e.g.,[128], Figure 5) can also act as inhibitors of ABCB1. The possibility of accommodating a range of compounds in different combinations reveals *broad binding regions*[127] with multiple anchor points (i.e., HBDs and unsaturated rings). Large ABCB1 binding regions were also observed by cryo-EM (e.g.,[71]).

If two identical molecules bind, the second inhibitory molecule has a lower affinity to the transporter than the activating first molecule[59]. Under most circumstances, "inhibition" is therefore a transient slowing-down of the transporter that rapidly fades away by dilution (see Supplementary Figure 4). This is in contrast to inhibitors of receptors (that work according to the lock-key principle). They generally show higher affinities to the receptor than the activators (see, e.g., the neurokinin-1 receptor. It binds its activator, substance P, an amphiphilic pain transmitter peptide, in the nanomolar concentration range[129] and inhibitors in the sub-nanomolar concentration range[130]).

The characteristic features of ABCB1 inhibitors

The principle feature of inhibitors is a very negative ΔG_{tr}° value. This is achieved with compounds exhibiting either a particularly high affinity to the transporter within the membrane, ΔG_{m}° (due to numerous HBAs such as cyclosporine A), or a very negative ΔG_{tr}° value (high logP or logD values) and an intermediate affinity to the transporter, ΔG_{m}° (e.g., encequidar, tariquidar, elacridar, zosuquidar, and OC144-093) [Table 1]. The sheer length and partial rigidity of some of the newer inhibitors may additionally impede rapid flopping.

A further feature inducing ABCB1 inhibition is the above-discussed inappropriate (e.g., DDM) or low amphiphilicity, which is quite common among inhibitors (e.g., cyclosporine A). A large list of inhibitors is given in a review by Artursson and colleagues[131]. In addition to many hydrophobic examples, dipyramidole, which is a relatively hydrophilic and non-amphiphilic compound, is listed as an ABCB1 inhibitor[131].

"Transport substrate or inhibitor"?

Alam and colleagues observed that zosuquidar ("inhibitor") and taxol ("substrate") bind to the same pocket and asked about "how ABCB1 distinguishes transport substrates from inhibitors and how these compounds exert opposite effects on the ATPase activity"[72]. These questions can be answered using the present data [Table 1]. Zosuquidar is very hydrophobic (high LogP) and has a rather small cross-sectional area perpendicular to the axis of amphiphilicity[132]. Thus it likely exhibits a very negative ΔG_{tr}°. Owing to the high membrane concentration, two molecules of zosuquidar will bind to the transporter already at low aqueous concentrations. In comparison, taxol and vinblastine exhibit rather large cross-sectional areas. Moreover, they are relatively hydrophilic and exhibit a much less negative ΔG_{tr}°. Thus, they will barely reach the concentration of half-maximum inhibition, K_2. The latter molecules also show a low flux across the membrane and are therefore prone to be exported by ABCB1 in cells (for vinblastine[78] and taxol[133]), which further enhances K_2.

Understanding ABCB1 inhibition is fundamental for understanding ABCB1 function. ABCB1 inhibition plays a significant and possibly underestimated role in drug-drug interactions resulting from polypharmacy[35]. Note, that systemic ABCB1 inhibition to enhance cancer drug absorption was not successful in clinical trials[134,135].

UNIDIRECTIONAL TRANSPORT CYCLE FOR ABCB1 ALLOCRITES

Combining the physicochemical insights gained in this review with our previous molecular dynamics simulations[33] yields the transport cycle for ABCB1 schematically summarized in Figure 7: (i) Allocrites partition into the lipid membrane, and accumulate in the cytosolic membrane leaflet with the polar part located in the interfacial membrane region. The polar part of the allocrite, carrying at least one type 1 pattern (with HBAs, see Figure 1), is attracted by the transporter and likely glides along the numerous HBDs at the protein surface towards the core of the membrane, where the attraction is highest, due to the

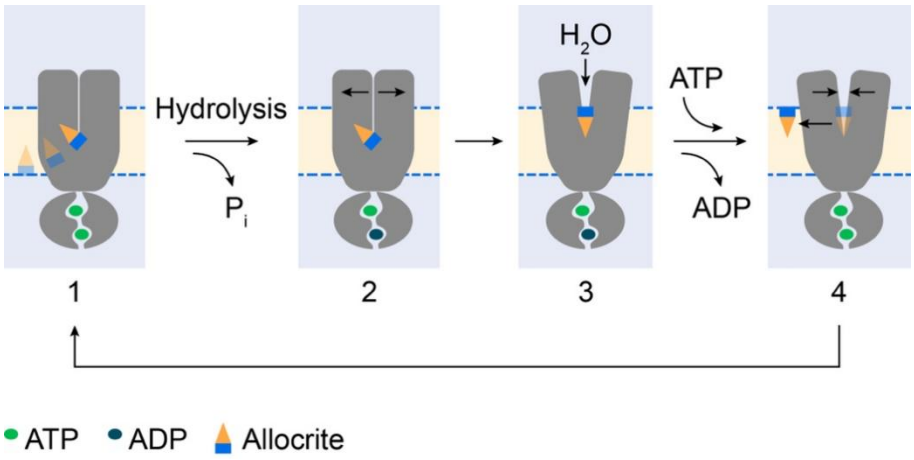

Figure 7. Unidirectional transport cycle in ABC exporters is driven by weak dipolar interactions between the allocrite and the transporter. (1) With two ATPs bound to the NBDs, the TMDs are in the outward closed resting state. The amphiphilic allocrite with HBAs in the polar part (blue) is attracted to the transporter via weak dipolar interactions. The polar groups are then drawn towards the center of the membrane ($\varepsilon_m \approx 2$). (2) Hydrolysis of ATP and release of inorganic phosphate (P_i) lead to an asymmetric occupation state of the NBDs, which initiates the opening of the TMDs. (3) The TMDs adopt an outward open conformation, which allows influx of water molecules. The allocrite flops (i.e., it turns around to expose its polar part towards the aqueous phase). The hydrogen bonds between the allocrite and the transporter are dissolved. The binding of ATP to the empty NBS restores the symmetric occupancy state and favors the outward closed conformation. The allocrites on the extracellular side of the cavity between the TMDs are squeezed out to the membrane (see also Ref.[33]).

lowest dielectric constant. (ii) Allocrite binding likely elicits a strain on the transporter that leads to ATP hydrolysis and opening of the transporter towards the extracellular side. (iii) Inflowing water molecules compete for dipolar interactions with the transporter and the allocrite and induce de-binding of the allocrite, a process described as the solvation exchange mechanism by Omote and Al-Shawi[136]. As water fills the newly formed cavity, allocrites orient with their polar groups towards the aqueous phase (i.e., they flop). (iv) Upon ATP rebinding, the transporter closes extracellularly. In this way, the potentially remaining allocrite is squeezed out of the transporter into the outer membrane leaflet. It then either diffuses into the aqueous phase or restarts the flip-flop cycle until it is degraded by other enzymes such as CYP3A4[137], attracting allocrites within the membrane by the same weak dipolar interactions. A more detailed description of the "*unidirectional ABCB1 transport cycle driven by weak dipolar interactions*," including supporting experimental data, is given in the Supplementary Materials. The rate-limiting step for the overall ATPase activity was previously suggested to be either the de-binding of the ligand or a conformational change of the enzyme, impeded by a bulky substrate[96]. We observed that the rates of ATPase activity and transport are lower for compounds with a higher affinity to the transporter, for more than one compound bound to the transporter, and for compounds of low amphiphilicity that are not easily oriented at a protein-water interface. Our findings thus suggest that the de-binding and flopping process is rate-limiting, supporting the early suggestions. A more detailed description, including experimental results, is given in the Supplementary Materials.

CONCLUSIONS

Thermodynamics: The *amphiphilicity* of allocrites[1] implies their accumulation in membranes. In the 1990s, ABCB1 was demonstrated to indeed attract its allocrites within the membrane. An allocrite recognition mechanism based on weak dipolar interactions that works in membranes was proposed. It explains the polyspecificity of ABCB1. With the advent of the first ABCB1 structures, the focus shifted to the protein *only*. In this context, the transporter was often treated conceptually as a receptor, binding its allocrites from the aqueous phase. With this review, we demonstrate that *understanding the nature of allocrite-ABCB1*

interactions requires inclusion of the transporter environment. The binding process starts with partitioning of the amphiphilic allocrite from the aqueous phase into the lipid membrane (step I). Partitioning is driven by classical hydrophobic interactions in the case of electrically neutral amphiphiles and by non-classical hydrophobic interactions in the case of cationic amphiphiles. Once in the membrane, the polar part of the allocrite (with HBAs) is attracted to the transporter (with HBDs and π-electron systems) (step II). Step I and Step II were quantified in terms of the free energies of binding ΔG_{part} and ΔG_{el}. The sum of these two free energies yields the overall free energy of allocrite binding from water to the transporter, ΔG_{bind}. Whereas partitioning (ΔG_{part}) *decreases* with increasing lateral packing density of the membrane, weak electrostatic attraction to the transporter (ΔG_{el}) was shown to *increase* with increasing lateral packing density and concomitantly decreasing dielectric constant of the membrane. Owing to these compensatory mechanisms regarding the membrane packing density, the concentration of half-maximum activation, K_1, changes only moderately in biological membranes.

Kinetics: P-glycoprotein was shown to alter the *membrane permeability*[1], which suggests the presence of two competing processes, passive diffusion into the cell and active transport out of the cell. Theoretically, these two competing processes can be described rather easily in different ways: Ref.[116] or Ref.[58]. Demonstrating these phenomena in a single experiment was more difficult and required ATPase activity measurements with large slowly diffusing allocrites in ABCB1-overexpressing cells as well as large slowly diffusing allocrites. These measurements revealed a significant concentration decrease in the cytosolic membrane leaflet (reflected by higher K_1 values than observed in inside-out plasma membrane vesicles of the same cells), which can be attributed to the flopping activity of ABCB1[69]. Combining the early drug efflux measurements[124] with ATPase activity measurements in the same cells, we obtained a stoichiometry of one ATP hydrolyzed per allocrite transported. Moreover, we highlight the importance of bell-shaped ATPase activity curves that account for binding of a second allocrite to the transporter[96,127]. The concept of two allocrites binding to ABCB1 is indispensable for understanding ABCB1 inhibition as well as drug-drug interactions.

Combining thermodynamics and kinetics yields insights into the nature of allocrite-transporter interactions: Generally, the steady-state ATP hydrolysis rate decreases with increasing allocrite affinity ΔG_{bind} to the transporter [Figure 5A]. However, a detailed inspection of Figure 5A shows that allocrites with identical free energies of binding ΔG_{bind} do not necessarily exhibit identical ATP hydrolysis rates. An allocrite undergoing weak, delocalized interactions with the transporter (e.g., via two HBAs and a weak charge) shows a *higher* steady-state ATP hydrolysis rate than a compound with a single strong point charge. Conversely, allocrites with inappropriate (e.g., DDM) or low amphiphilicity (e.g., cyclosporin A[79] and some of the newer inhibitors such as elacridar) show *lower* steady-state ATP hydrolysis rates than expected from their ΔG_{bind} value. A substantial *increase* in the steady-state ATP hydrolysis rate is observed if ABCB1 is reconstituted in detergent micelles. Owing to the higher curvature and lower packing density, micelles exhibit higher dielectric constants than bilayers in lipid vesicles, which in turn significantly reduce dipolar interactions. Micelles, moreover, exhibit a lower "reservoir capacity for amphiphiles" (i.e., a negligible "ΔG_{part}"). Therefore, allocrites that appear as inhibitors in membranes appear as activators in micelles. ABCB1 is thus a robust transporter (or rather a floppase) that perfectly adapts to membranes or micelles exhibiting very different lateral packing densities. The increase in *net permeability* in loosely packed systems is thus not due to a deficient transporter, but to the strongly enhanced passive diffusion. The clear dependence of the steady-state ATP hydrolysis rate on the nature and number of dipolar interactions, as well as on the dielectric constant of the lipid or micellar environment, points to a flopping process that, at least initially, takes place at *the interface between the lipid membrane and the protein*. The effects observed do not seem possible either

at a protein-water interface (as suggested in alternating access models) or inside a protein channel, which is completely shielded from the lipid membrane.

Combining the physical chemical insights gained in this review with our previous molecular dynamics simulations[33] suggests that the ABCB1 transport cycle, including allocrite recognition, binding, and transport, is driven by weak dipolar interactions. Allocrite binding induces the hydrolysis of one ATP molecule, which leads to transporter opening towards the extracellular side. Influx of water molecules allows for allocrite flopping. ATP rebinding re-closes the transporter at the extracellular side and expels the potentially remaining allocrites. The individual steps can be quantified when taking into account that the transporter was optimized to operate in a membrane.

DECLARATIONS

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Authors' contributions

Wrote the manuscript: Seelig A

Provided Figure 4 and Tables, read and commented on the manuscript at different stages, read and approved the final manuscript: Li-Blatter X

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Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Commentary

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Concomitant medications and circulating tumor cells: friends or foes?

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Abstract

The use of concomitant medications by patients with cancer is observed almost globally; however, little attention has been paid to this topic in the medical literature. Most clinical studies do not describe the type and duration of drugs used at the time of inclusion and during treatment or how these drugs may affect the experimental and/or standard therapy. Even less information has been published on the potential interaction between concomitant medications and tumor biomarkers. However, we do know that concomitant drugs can complicate cancer clinical trials and biomarker development, thus contributing to their interaction, leading to side effects, and resulting in suboptimal adherence to anticancer treatment. On the basis of these premises and moving from the study by Jurisova *et al.*, which reported the effect of commonly used drugs on the prognosis of women with breast cancer and the detection of circulating tumor cells (CTCs), we comment on the role of CTCs as an emerging diagnostic and prognostic tool for breast cancer. We also report the known and hypothesized mechanisms of CTC interplay with other tumor and blood components, possibly modulated by widespread drugs, including over-the-counter compounds, and discuss the possible implications of commonly used concomitant medications on CTC detection and clearance. After considering all these points, it is conceivable that concomitant drugs are not necessarily a problem, but on the contrary, their virtuous mechanisms can be exploited to reduce tumor spread and enhance the effect of anticancer therapies.

Keywords: Breast cancer, concomitant medications, circulating tumor cells

Identifying and isolating circulating tumor cells (CTCs) is the most straightforward method to obtain insight into the progression of a neoplastic disease at a systemic level without using invasive methods. However, today, almost three decades after this brilliant consensus was reached, dealing with CTCs remains complicated by technical difficulties and limited clinical utility[1]. An increasing number of studies have been published on CTCs that address all aspects of this biomarker development (technical validity, clinical validity, and clinical utility). In 2004, the United States Food and Drug Administration (FDA) approved CellSearch™ CTC counting for staging patients with metastatic breast, prostate, and colorectal cancers[2]. More recently, several cutting-edge studies have reported valuable information obtained from genomic, transcriptomic, and epigenomic analyses on single isolated CTCs[3]. However, many uncertainties remain in the CTC domain, including the mechanisms underlying their release, clearance, biological significance (i.e., association with the origin, aggressiveness, and metastatic potential of tumors) in addition to response to treatment.

In their study, Jurisova *et al.* addressed an additional neglected aspect: the impact of concomitant medications on the detection of CTC[4]. Concomitant medications include prescription drugs, over-the-counter drugs, or dietary supplements taken by an individual at the time of diagnosis and/or treatment of a specific disease. Patients with cancer are more likely to take concomitant drugs compared with other patients for two main reasons: first, to minimize disease-related symptoms, and second, as an elective remedy for the disease itself, such as in the case of self-prescribed herbal products[5]. Concomitant drug use is therefore almost universally observed among patients with cancer, occurring in up to 99% of all cases, whereas it is merely widespread in other types of diseases, ranging between 70% and 83%[6]. Concomitant drugs may interact with anticancer drugs and have an effect on outcomes, resulting in erroneous conclusions, which is why the rules of Good Clinical Practice require physicians to pay attention to concomitant drugs used before and during a specific treatment[7].

Because CTCs are used for prognosis as well as for monitoring treatment response, Jurisova *et al.* investigated the possible associations between the use of chronic medications and long-term survival and attempted to avoid the confounding factors caused by the disease itself[4]. Importantly, they used a CTC-detection approach that can distinguish CTCs with a frankly epithelial phenotype (referred to as CTC_EP) from those undergoing epithelial-mesenchymal (E/M) transition (EMT) and loss of epithelial protein markers and genes expression (referred to as CTC_EMT). Such a distinction would not have been possible using the FDA-approved CellSearch™ approach, which only enumerates the CTC-expressing epithelial markers (EpCAM and CKs). It should be noted that the EMT is not a binary process. Some cells may achieve a hybrid E/M phenotype or migrate as CTC clusters[8]. The ability to attain a hybrid E/M phenotype rather than a full EMT phenotype confers increased metastatic potential, and CTC clusters are observed more frequently in the early stage rather than during the metastatic stage[9]. However, long-term treatments, such as anticancer treatments, are known to induce EMT programs, which are often associated with the resistance mechanism, as phenotypic plasticity confers invasiveness and increases the dissemination potential[10]. In this case, what is actually a resistance pattern driven by EMT could instead be misinterpreted as a reduction or disappearance of epithelial CTC (and thus considered a treatment response). This phenomenon has been described as "drug-related undetectable CTC status", and it has been ascribed to the hypoxia-driven EMT triggered by the antineoangiogenic drug bevacizumab[11]. Researchers have also described a similar effect in patients treated with the monoclonal antibody denosumab[12].

Luckily, Jurisova *et al.* relied on the detection of both CTC_EP and CTC_EMT in a cohort of > 400 women with stage I-III breast cancer who had complete medical history and information on the use of concomitant medications available[4]. Thus, they dodged the problem of misinterpreting the absence of CTC as a sign of

absence of systemic dissemination and/or of good prognosis simply owing to the technical failure in detecting CTC_EMT. In addition, 10% of patients had primary tumors lacking hormone receptors, which are basal-like in most cases and rarely express CK19, instead showing high expression of CK5 and CK17[13]. Increased CTC_EMT positivity rates were consistently observed for all classes of drugs tested, although none reached statistical significance. In contrast, the use of angiotensin-converting enzyme (ACE) inhibitors was associated with an almost 50% decrease in CTC_EP positivity. The clinical value of interfering with the angiotensin/renin system has rarely been addressed in the literature and has produced controversial results. Specifically, the landmark LACE study reported in the last decade stated that patients with breast cancer undergoing treatment with ACE inhibitors were more likely to experience recurrence compared with nonusers[14]. Conversely, another study that investigated the impact of ACE inhibitors in the setting of preoperative treatment reported no immediate effects on tumor response but did find an improvement in relapse-free survival[15]. More recently, researchers have suggested that ACE inhibitors have no direct cytotoxicity against tumor cells but rather show the potential to inhibit tumor growth owing to the suppression of vascular endothelial growth factor-induced angiogenesis *in vivo*[16]. Thus, we could hypothesize that ACE inhibitors exert an antiangiogenic activity that hinders the extravasation of CTCs and/or the formation of a metastatic colony in distant organs; alternatively, they could induce tumor mass dormancy or prolong dormancy maintenance. Taken together, these findings suggest that medical oncologists should interpret the results of CTCs on the basis of an individual's use of ACE inhibitors. Furthermore, the findings also have an impact on pooled data collected in clinical studies, suggesting that CTC results should be evaluated separately in users and nonusers of ACE inhibitors.

Drugs other than antihypertensives have been studied with respect to their prognostic effect in cancer, and specifically on the CTCs, which are potential therapeutic targets not only individually but also in aggregated forms with other cell types. For example, in animal models, anti-aggregation therapy can potentially reduce the risk of tumor metastases. Li *et al.* explored genetically modified platelets expressing tumor necrosis factor-related apoptosis-inducing ligand and found that blocking the interactions between platelets and CTCs reduced metastatic potential in a prostate cancer model[17]. In addition, platelets expressing tumor necrosis factor-related apoptosis inducing ligand could significantly eradicate cancer cells *in vitro*. Thus, agents that are not strictly anticancer could play a virtuous role in CTC interactions. In fact, clinical evidence has shown that aspirin reduced all-cancer mortality after only 5 years[18]. Those results can hardly be interpreted as aspirin only affecting carcinogenesis or early cancer growth and instead suggest that it may act as an inhibitor of cancer metastasis. Finally, treatment with the salicylate diflunisal induced disease remission (complete and partial) in two patients who refused standard treatment. Notably, both responders showed reduced post-treatment CTC levels[19]. The most accepted hypothesis is that the downregulation of the platelet-related COX-1 pathway by aspirin may explain the EMT of CTCs and contribute to its antimetastatic effects.

CTCs can also form heteroclusters with neutrophils, and this increases their metastatic potential[20]. Preclinical studies in mouse models have demonstrated that the anti-inflammatory zileuton, an inhibitor of arachidonate 5-lipoxygenase (5-LOX) and thus of leukotriene production, reduces spontaneous metastasis[21]; more recently, zileuton-loaded nanoparticles have been shown to reduce breast CTCs[22]. Interestingly, among 5-LOX inhibitors, there is curcumin, which is often used as a food supplement in general as well as in cancer patient populations[23]. However, Jurisova *et al.* did not observe any effects of nonsteroidal anti-inflammatory drugs, probably because of the low number of patients reporting the regular use of such medications[4]. All of these data are limited by the low number of patients and are, in some cases, purely anecdotal. Nevertheless, they offer the opportunity to hypothesize that CTCs not only may be used as a surrogate marker of tumor response but also may themselves become a treatment target with therapies

aimed at decreasing their survival in the circulation and tissue homing.

Similarly to the CTC/platelets and CTC/neutrophils heterogroups, CTC clusters are central to metastatic potential[24]. Preclinical data showing that the Na^+/K^+-ATPase inhibitors digoxin and ouabain used as antiarrhythmic agents are able to disaggregate CTCs clusters[25] led to the ongoing "Digoxin Induced Dissolution of CTC Clusters" trial (NCT03928210), the results of which are urgently awaited.

Studies such as the one reported by Jurisova *et al.* contribute to the CTC field because even slight effects on CTC enumeration can hide interesting mechanisms exerted by common drugs that can be a premise to their repurposing or off-target use[4].

DECLARATIONS

Authors' contributions

Equally contributed to discussing and writing this commentary: Di Cosimo S, Cappelletti V

Availability of data and materials

Not applicable.

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Commentary

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New perspectives on epigenetic modifications and PARP inhibitor resistance in HR-deficient cancers

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Abstract

The clinical treatment of DNA-repair defective tumours has been revolutionised by the use of poly(ADP) ribose polymerase (PARP) inhibitors. However, the efficacy of these compounds is hampered by resistance, which is attributed to numerous mechanisms including rewiring of the DNA damage response to favour pathways that repair PARP inhibitor-mediated damage. Here, we comment on recent findings by our group identifying the lysine methyltransferase SETD1A as a novel factor that conveys PARPi resistance. We discuss the implications, with a particular focus on epigenetic modifications and H3K4 methylation. We also deliberate on the mechanisms responsible, the consequences for the refinement of PARP inhibitor use in the clinic, and future possibilities to circumvent drug resistance in DNA-repair deficient cancers.

Keywords: Double strand break repair, histone methylation, PARP inhibitor, resistance, SETD1A, BOD1L, H3K4

CANCER THERAPY AND DOUBLE STRAND BREAK REPAIR

Many cancer patients will receive radiotherapy as part of their treatment[1] which relies on ionising radiation (IR) to induce highly toxic lesions in the form of chromosomal DNA double-strand breaks (DSBs). DSBs represent the most lethal type of DNA damage induced by genotoxic therapy, but their programmed repair have important physiological roles in normal metabolism and immune system development. Repair of DSBs

is also essential to maintain genome stability and therefore represents a vital anti-tumour barrier[2].

There are two main pathways used by cells to ensure the efficient repair of DSBs, non-homologous end joining (NHEJ) and homologous recombination (HR). The choice between these two repair pathways is tightly regulated by numerous mechanisms including the cell cycle, post-translational modifications of DNA repair proteins, and interactions between DNA repair proteins and chromatin[3]. NHEJ is the principal DSB repair pathway and is responsible for repairing 85% of all DSBs induced by IR[4]. Although NHEJ is active in all phases of the cell cycle, it predominates in G1, and involves direct ligation of the two broken DNA ends. In contrast, HR requires a homologous sister chromatid as a repair template and is therefore restricted to the S and G2 cell cycle phases. HR also requires DNA end resection, which is carried out by a number of cellular nucleases including MRE11, CtIP, EXO1 and DNA2[5,6]. The actions of these proteins results in formation of a 3' ssDNA tail, which is then coated with the single-stranded DNA binding protein RPA (Replication Protein A) which acts as a substrate for RAD51-mediated homology search and strand invasion. In addition to these two classical DSB repair pathways, alternative mutagenic DSB repair mechanisms have been identified. These include microhomology-mediated end joining (MMEJ; also known as alternative end-joining) and single strand annealing (SSA)[7]. As with HR, these alternative pathways rely on extensive end resection. However, lack of a repair template results in significant loss of genetic information in these pathways, therefore MMEJ and SSA are considered error-prone and highly mutagenic.

BRCA1 AND 53BP1: BALANCING DSB REPAIR

The choice between HR and NHEJ is controlled by multiple factors, of which 53BP1 and BRCA1 are two of the most important. The antagonistic relationship between 53BP1 and BRCA1 controls DNA-end resection and thus dictates repair pathway choice. 53BP1 is one of the first proteins recruited to DSBs, which is mediated by the interaction between 53BP1 and two histone modifications: H4K20me2 and H2AK15Ub. Localisation of 53BP1 at DSBs protects DNA from resection via a series of downstream effectors including PTIP[8], RIF1[9-12], REV7[13,14], and the Shieldin -CST- polα complex[15-18]. This pathway inhibits the localisation and activity of BRCA1 and the endonuclease CtIP to DSBs, promoting NHEJ, and maintains DSBs via fill-in of ssDNA.

In contrast, the tumour suppressor BRCA1 promotes end-resection in S and G2 and counteracts 53BP1. In part, this is mediated by post-replicative dilution of H4K20me2 on "parental" histones, promoting the recruitment of BRCA1 and its partner BARD1 to DSBs where it displaces 53BP1 from DNA ends[19,20]. BRCA1 also facilitates end-resection and therefore HR by promoting the actions of phosphorylated CtIP and MRE11[21]. In addition, BRCA1 has further roles in HR, promoting recruitment of the RAD51 recombinase to ssDNA. BRCA1 also interacts via PALB2 with BRCA2, with this tripartite complex assisting RAD51 loading and recombination[22]. Finally, BRCA1 and BARD1 enhance the recombinase activity of RAD51, promoting successful HR repair.

HISTONE METHYLATION AND DNA REPAIR

Histone lysine methylation is a critical post-translational modification essential for numerous cellular processes. This modification is carried out on lysine residues within histone tails by a family of enzymes known as lysine methyltransferases (KMTs). In terms of DNA repair, several lysine methylation events are known to be required for the proper repair of DSBs[23]. For example, the localisation of 53BP1 to damaged chromatin requires binding of its tandem Tudor domains to di-methylated lysine 20 of histone 4 (H4K20me2)[24-26]. Furthermore, the binding of BARD1 to di-methylated H3K9 (H3K9me2) is required to retain BRCA1 at DSB sites to promote repair by HR[27]. These examples illustrate the importance of histone methylation events in the regulation and recruitment of proteins critical for DSB repair.

Methylation of lysine 4 of histone H3 (H3K4me) is most well-known as a marker for genomic regions undergoing active transcription. In yeast, H3K4me is carried out by a single methyltransferase, Set1, but in higher eukaryotes this modification is principally catalysed by the KMT2 family of enzymes[28]. Studies have shown that transcription is required for DSB repair and suggested that without active transcription DNA damage response (DDR) proteins are unable to efficiently localise to repair foci[29-31]. However, transcription can also be largely supressed at sites of DSBs despite the presence of H3K4me[32], suggesting that this histone modification could also have an important transcription-independent role in DNA repair.

Several studies have examined levels of H3K4me at DSBs, yielding conflicting results. Globally, there appears to be no change in the levels of H3K4me3 following DNA damage when examined by immunoblotting[33,34]. However, more sensitive methods have demonstrated differences in the prevalence of this modification at DSBs. Several studies suggest that H3K4 di-and tri-methylation levels decrease following UV laser micro irradiation or in GFP-based DSB repair reporter assays. This is attributed to increased activity of various lysine demethylases (KDMs) that act on H3K4, including KDM1A, KDM5A and KDM5B[35-37]. In contrast, other studies demonstrate an increase in H3K4me3 at DSBs, the removal of which by KDM5B is required to allow recruitment of DNA repair factors[38]. We recently used chromatin immunoprecipitation (ChIP) to measure levels of histone methylation surrounding newly-formed DSBs induced on a Lac-operator by mCherry-lacI-FokI. These studies revealed an increase in H3K4me3 following DSB induction[39]. Collectively these data all indicate an important role for H3K4me in DSB repair, however their conflicting findings suggest that results could be dependent upon type of DNA damage induced and the methods used to detect this modification. Interestingly, H3K4 methylation is also important for other types of DNA repair, as loss of H3K4me at replication forks during replication stress induces genome instability by allowing degradation of DNA[40].

H3K4 METHYLATION AND HR/NHEJ

Analysis of DSBs undergoing repair by NHEJ or HR (classified by the proteins bound to these breaks) first identified that HR-competent chromatin is enriched in H3K4me2[41]. In support of this, favouring HR-mediated repair by treating cells with an inhibitor of DNA-PKcs increases levels of H3K4me at DSBs induced by the yeast rare-cutting endonuclease ISceI[42]. These studies on regions of "open" chromatin initially suggested that H3K4me may promote HR-mediated repair.

Recently, we have significantly revised thFeither BRCA1 or BRCA2 are associated with susceptibility to multipleese findings by identifying an important role for H3K4 methylation in facilitating RIF1-dependent NHEJ[39]. We showed that loss of SETD1A, a member of the KMT2 family of methyltransferases, or its cofactor BOD1L, significantly impairs RIF1 localisation to DSBs and their subsequent repair by NHEJ. Loss of SETD1A/BOD1L function induced uncontrolled DNA end resection, impaired end-joining of dysfunctional telomeres, and reduced immunoglobulin class switching, all of which are characteristic of 53BP1-RIF1 deficiency[10]. This is dependent upon lysine methylation by SETD1A, as these phenotypes were also apparent in cells deficient in SETD1A activity, in H3K4 methylation or overexpressing the H3K4 demethylase KDM5A. Furthermore, RIF1 and H3K4me3 overlap at a genome wide level, which seems independent of external factors including origin firing or transcription start sites. Therefore, H3K4 methylation seems to directly stimulate DSB repair by NHEJ. Interestingly, our data suggests that the mechanism by which H3K4me controls DSB repair is direct, as *in vitro* binding assays showed that RIF1 binds directly to methylated H3K4, an interaction mediated by the HEAT repeats present in the N-terminal of RIF1[39]. This is particularly intriguing given that similar experiments demonstrate that BRCA1 binds with a higher affinity to unmethylated H3 peptides compared to H3K4me3 peptides[36], suggesting that H3K4me at DSBs might directly influence DSB pathway choice by regulating both BRCA1 and RIF1. Despite these

advances, it is unclear exactly how H3K4me determines if a DSB undergoes repair by HR or NHEJ, and much work remains to identify the specific mechanism(s).

TARGETING HR DEFICIENCY WITH POLY (ADP-RIBOSE) POLYMERASE INHIBITORS

Inherited mutations in either BRCA1 or BRCA2 are associated with susceptibility to multiple cancer types including a higher risk for breast and ovarian cancer. Since BRCA1 and BRCA2 regulate multiple stages of HR, cells with compromised BRCA1/BRCA2 activity are deficient in HR activity[43]. Targeting DSB repair deficiency represents an important paradigm in cancer therapy, exemplified by the use of poly (ADP-ribose) polymerase inhibitors (PARPi) to treat HR-deficient tumours[44]. PARPi work by trapping PARP enzymes on DNA, preventing the repair of single strand breaks (SSBs) via a PARP-reliant pathway known as base excision repair. As a consequence, unrepaired SSBs are converted into DSBs when encountered by replication forks. Since the resulting DSBs require repair via HR, in cells lacking sufficient levels of BRCA activity these DSBs cannot be repaired, resulting in NHEJ-dependent toxic chromosome fusions which drive cell death[44,45]. To date, four PARP inhibitors have received clinical approval in multiple BRCA1- and BRCA2-deficient settings: olaparib, rucaparib, talazoparib and niraparib.

Although treatment with PARPi induces a significant increase in patient survival, many patients develop resistance, and their prognosis is poor. Indeed, 40% of metastatic breast cancer patients harbouring germline BRCA1/2 mutations failed to respond to olaparib[46]. This resistance seems to arise from 4 main biological mechanisms[47]: restoration or reactivation of BRCA1 or BRCA2 activity (e.g., by reversion mutations or promoter demethylation); loss of PARP1 or PARG expression; upregulation of PARPi efflux; and rewiring of the DDR, including restoration of HR and replication fork protection. In particular, loss of members of the 53BP1-dependent NHEJ pathway (e.g., RIF1, REV7, 53BP1, Shieldin) renders BRCA1-deficient cells resistant to PARPi[9,13,17,21]. This is thought to be mediated via the absence of the Shieldin complex on DNA ends, leaving them unprotected and subject to resection by nucleases to initiate repair by HR[48]. Therefore, the balance between HR and NHEJ is key in determining the response to these targeted inhibitors. Interestingly, this mechanism of resistance has not been observed in BRCA2-deficient cells to date, which is likely due to differing roles between BRCA1 and BRCA2 in promoting HR[49].

SETD1A AND H3K4ME IN PARP INHIBITOR RESISTANCE

Our recent findings impact substantially on these mechanisms of drug resistance. We demonstrated that, like loss of RIF1[10], loss of SETD1A also induces PARPi resistance in BRCA1-deficient cells[39]. Our data also demonstrate that this resistance can be linked to a partial restoration of HR in these cells, as we observed cells deficient in both BRCA1 and SETD1A were able to recruit RAD51 to chromatin following treatment with PARPi, and that functional HR was at least partially restored in cells lacking both BRCA1 and SETD1A. Therefore, loss of SETD1A allows reactivation of HR in BRCA1-deficient cells [Figure 1]. Strikingly, many of these phenotypes (increased end-resection, defective RIF1 recruitment, PARPi resistance) were also observed in cells expressing SETD1A but in which H3K4 methylation had been perturbed by either mutation or over-expression of a lysine demethylase[39], suggesting that PARPi resistance in BRCA1-defective cells is driven by epigenetic modifications, at least in part. Indeed, given that RIF1 interacts with H3K4me3 *in vitro*, this suggests that SETD1A-mediated histone methylation is responsible for promoting NHEJ and therefore sensitivity to PARPi [Figure 1].

Interestingly, SETD1A, H3K4me and RIF1 dysfunction is linked with a second mechanism known to control PARPi resistance, the protection of nascent DNA[40,50]. Newly replicated DNA is protected from degradation by several factors, including BRCA1, BRCA2, RIF1, 53BP1, SETD1A, BOD1L and H3K4me[51], all of which also have roles in DSB repair. At stalled replication forks, these factors act to suppress the

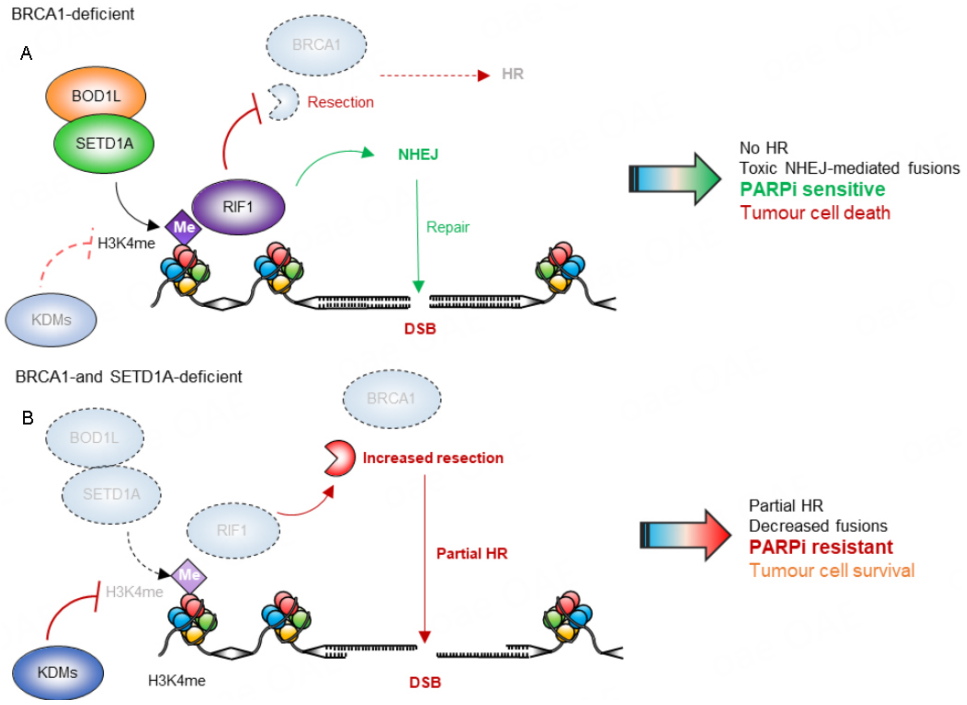

Figure 1. Effects of BOD1L/SETD1A loss on PARP inhibitor sensitivity in BRCA1-deficient cells. (A) H3K4me mediated by the BOD1L/SETD1A complex promotes RIF1 localisation at DNA double-strand breaks (DSBs) and stimulates NHEJ. In BRCA1-deficient cells, DNA-end resection and RAD51 loading are inhibited and lesions cannot be repaired by homologous recombination (HR), resulting in sensitivity to PARP inhibition and cell death. (B) Depletion of the BOD1L/SETD1A complex results in loss of H3K4me and decreased RIF1 localisation to DSBs. This allows DNA end-resection and RAD51 loading, partially restoring HR. This mediates resistance to PARP inhibition and allows cells to survive. Me: Methylation; PARP: poly(ADP) ribose polymerase; NHEJ: non-homologous end joining; KDMs: lysine demethylases.

actions of nucleases including DNA2, EXO1 and MRE11. In their absence, excessive nucleolytic degradation leads to genomic instability and drives sensitivity to PARPi. Therefore, loss of fork degradation (or restoration of protection) leads to PARPi resistance[52,53]. However, this only seems to be applicable in certain genetic backgrounds: whilst loss of BRCA1/2 and thus loss of fork protection sensitises cells to PARPi, cells deficient of SETD1A, RIF1 or 53BP1 are also deficient in this pathway, but are not sensitive to PARPi. Furthermore, co-depletion of SETD1A and BRCA1, or RIF1 and BRCA1, does not restore fork protection[39], suggesting a complex interplay between roles for these proteins at DSBs *vs.* replication forks. Nevertheless, our findings that PARPi sensitivity can be driven by epigenetic changes are in broad agreement with other studies demonstrating that such modifications can also regulate the response to PARPi[53,54]. Clearly, much more work remains to be done to comprehend the different mechanisms of DDR rewiring and how these impact on PARPi resistance in various genetic backgrounds and tumour types.

THERAPEUTIC IMPLICATIONS AND FUTURE PERSPECTIVES

Identifying resistance: In terms of clinical implication, predicting which patients may develop resistance to PARPi is an important area of investigation. Previous work has shown that significant changes in the expression and activity of methyltransferase and demethylase enzymes occurs during cancer development, suggesting that disruption to their function is important in disease pathogenesis[55]. Furthermore, analysis of publicly available datasets suggests that SETD1A expression correlates with chemotherapeutic sensitivity and overall survival in multiple tumour types[56,57]. This provides further evidence that SETD1A expression might be a useful prognostic indicator to be considered when choosing a patient's treatment regime

[Figure 1]. Furthermore, monitoring SETD1A expression during the onset of resistance, and linking BRCA1 mutation status with SETD1A expression, would be invaluable in evaluating its utility as a potential biomarker. Taken together, profiling of SETD1A expression may well be a valuable prognostic tool to identify patients who are more likely to develop resistance to PARPi allowing them to be placed on alternative therapies including KDM inhibitors or in combination other genotoxins, in the hope that this would kill resistant cancer cells.

Therapeutic approaches: Investigating novel ways of manipulating DSB repair is crucial for the development of new and more effective treatments for patients treated with PARPi [Figure 2]. Several potential strategies could be envisaged to prevent HR reactivation upon PARPi-resistance, ultimately increasing the efficacy of these therapies. Firstly, increasing H3K4me could represent a direct approach to facilitate RIF1 recruitment to DSBs, promoting NHEJ and driving toxic chromosomal fusions and cell death. This could be achieved via manipulating SETD1A expression/activity, or the inhibition of KDM enzymes to prevent the removal of specific methylation marks. KDM1A/LSD1 is a prominent demethylase which counteracts the activities of SETD1A, and inhibitors to this protein have already been developed[58,59] and are currently being assessed for their use in cancer therapy. This raises the possibility that alleviating PARPi by manipulating the balance between H3K4 methylation and demethylation using these inhibitors could offer potential treatment benefit. Further pre-clinical work leading to their exploration in BRCA1-deficient patients would be an exciting avenue of future research.

A second approach to prevent reactivation of HR would be to inhibit the cellular nucleases responsible for DNA resection [Figure 2]. Loss of the 53BP1 pathway and/or SETD1A in BRCA-deficient cells allows uncontrolled end-resection by nucleases such as MRE11, CtIP, EXO1 and DNA2[39]. Combining PARPi treatment with inhibitors of these nucleases could be a promising way of preventing HR reactivation. Indeed, there is already evidence from pre-clinical studies that MRE11 inhibitors sensitise cancer cells to other agents such as IR[60]. Furthermore, MRE11 activity determines the sensitivity of cells to PARPi treatment in colorectal cancer[61]. However, given the diverse roles of MRE11 it would be important to monitor effects of its inhibition to ensure functions aside from its role in DNA end-resection are not compromised. CtIP depletion has also been shown to sensitise breast[62] and ovarian[63] cancer cells to treatment with PARPi, however this appears independent of BRCA-deficiency. As above, this opens novel areas of exploration, and could provide benefit to treat tumours without the traditional "BRCA-deficient" definitions.

Thirdly, deficiencies in pro-NHEJ components drive PARPi resistance in BRCA1-deficient cells, but also induces collateral vulnerabilities to other DNA-damaging agents including IR and cisplatin[17]. Exploring how loss of SETD1A/H3K4me affects the response to other genotoxic agents may help to identify other therapies that could be used to bypass PARPi resistance [Figure 2]. Notably, loss of SETD1A or its cofactor BOD1L sensitise cells to inter-strand crosslink (ICL)-inducing agents similar to cisplatin[40]. Furthermore, combinations of PARPi with pharmacological inhibitors to histone deacetylases, apical DNA repair kinases ATM and ATR, PI3K and mTOR, and immune checkpoint proteins have all been studied extensively[64], and may provide worthwhile avenues of investigation in cells lacking SETD1A, BOD1L or H3K4me. Finally, exploring the link between SETD1A and H3K4me with MMEJ may offer an alternative therapeutic vulnerability[65].

Beyond BRCA: There is growing evidence that the efficacy of PARPi as an anti-cancer therapy extends beyond BRCA1/BRCA2-deficiency to a range of other factors involved in HR. For example, loss of other key HR pathway proteins such as RAD51 and PALB2, as well as the apical DNA repair kinase ATM, gives

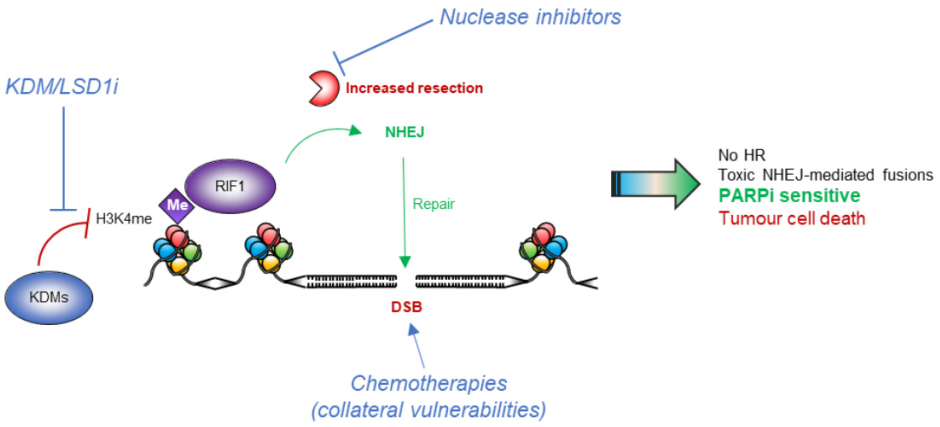

Figure 2. Future strategies to restore PARP inhibitor efficacy in BRCA1-deficient cells upon loss of SETD1A function. Sensitivity to PARP inhibition following loss of the BOD1L/SETD1A complex or H3K4me perturbation could potentially be restored via 3 mechanisms including: (1) inhibition of the lysine demethylases (KMD5 and LSD1) responsible for removing H3K4me; (2) inhibiting nucleases such as MRE11 to prevent DNA-end resection which facilitates HR; and (3) exploiting collateral vulnerabilities using chemotherapeutics, e.g., cisplatin. Me: Methylation; PARP: poly(ADP) ribose polymerase; HR: homologous recombination; NHEJ: non-homologous end joining; DSB: double-strand break; KDMs: lysine demethylases.

rise to synthetic lethality with PARP inhibition[66]. However, the mechanisms of resistance applicable to these contexts have not been widely explored. Previous studies have indicated that DDR rewiring is unable to restore HR in BRCA2-deficient cells. For example, depletion of 53BP1 cannot rescue HR in BRCA2-deficient mouse embryonic fibroblasts[49]. To date, known PARPi resistance mechanisms in BRCA2-deficient cells include loss of the PARG glycosylase[67] and restoration of functional BRCA2 activity via the acquisition of secondary reversion mutations[68]. This suggests that the resistance mechanisms acting in BRCA2-deficient cells differ significantly to those in other HR-deficient contexts. A key area for future investigation is therefore to establish whether PARPi resistance induced by loss of SETD1A provides a general mechanism of resistance that can be applied to wider HR-deficient contexts including RAD51, ATM and possibly BRCA2 deficiency. Combined with the above developments, this will increase the efficacy of DDR inhibitors in the clinic and help develop novel biomarkers and treatment strategies to overcome resistance.

CONCLUSION

The induction of DSBs by chemo- and radio-therapy has been used for many years in order to successfully treat a range of different cancers. However, the one major disadvantage of this approach is its lack of specificity. More recent developments involving the use of targeted inhibitors of DSB repair pathways such as PARPi have enabled more selective targeting of cancer cells, exploiting their intrinsic vulnerabilities such as HR deficiencies in BRCA-mutated cancers. However, these approaches are hampered by resistance. Our recent findings[39] have added to this field by identifying the potential clinical usefulness of regulating RIF1-dependent NHEJ through manipulation of SETD1A-dependent H3K4me. This is of particular relevance in BRCA1-deficient patients who develop PARPi resistance in the clinic, as maintaining H3K4 methylation/SETD1A activity and therefore the recruitment of RIF1 to DSBs could be a key strategy to prevent treatment resistance in these patients. Despite these advances, there is still much work to be done in the fields of SETD1A, NHEJ and histone methylation to enable the development of more tailored treatments to eradicate human cancers.

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Authors' contributions

Made substantial contributions to the conception, design, writing and editing of the manuscript: Bayley R, Sweatman E, Higgs MR

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Opinion

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The change of paradigm in the treatment of HER2-positive breast cancer with the development of new generation antibody-drug conjugates

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Abstract

HER2-positive breast cancer is an aggressive disease. As a result of the development of specific HER2-targeted therapies, such as trastuzumab, more than 20 years ago, the prognosis of these patients has improved. Metastatic HER2-positive breast cancer patients are achieving better survival rates upon treatment with anti-HER2 therapies than patients with HER2-negative disease. Double HER2 blockade with trastuzumab and pertuzumab combined with a taxane achieved an unprecedented survival of over 57 months in first-line patients. Trastuzumab emtansine, the first antibody-drug conjugate approved for patients in second-line treatment was a potent cytotoxic agent bound to trastuzumab and is currently a standard therapeutic strategy. Despite the progress in treatment development, most patients develop resistance and eventually relapse. Advances in the design of antibody-drug conjugates have led to the development of new generation drugs with enhanced properties, such as trastuzumab deruxtecan and trastuzumab duocarmazine, which are significantly changing the paradigm in the treatment of HER2-positive metastatic breast cancer.

Keywords: Breast cancer, HER2-positive, ADCs, New drugs, Mechanisms of resistance

INTRODUCTION

The development of anti-HER2 therapies: from antibodies to antibody-drug conjugates

Human epidermal growth factor receptor 2 protein (HER2)-positive breast cancer is an aggressive disease that accounts for approximately 15%-20% of the total breast cancer cases worldwide[1]. Most of these cases are diagnosed at the early stage. The HER2 pathway drives the growth and expansion of tumor cells. Research into the development of anti-breast cancer therapies has been focused on blocking the HER2 receptor with different strategies.

Trastuzumab

Trastuzumab is an anti-HER2 humanized monoclonal antibody directed against the extracellular portion of HER2. Trastuzumb was the first therapy approved by the FDA in 1998 for metastatic breast cancer patients with tumors with HER2 overexpression and it was approved in 2006 for use in the adjuvant setting after demonstrating a significant benefit in progression-free survival (PFS) and overall survival (OS) in both early and advanced disease[2,3]. The mechanisms of action of trastuzumb are the inhibition of HER2 shedding and inhibition of the PI3K-AKT pathway, resulting in an attenuation of cell signaling and promotion of antibody-dependent cell-mediated cytotoxicity (ADCC). Trastuzumab has significantly changed the landscape for HER2-positive patients[4]. However, some tumors acquire resistance to trastuzumab through mechanisms including increased cell signaling, PTEN loss, PIK3CA mutations, increased AKT activity, alternative cell signaling mediated by EGFR pathways, TGF-α overexpression, and expression of extracellular domain-truncated HER2 (p95 HER2)[5].

Pertuzumab

To overcome trastuzumab resistance and improve treatment efficacy, a strategy was developed of combining trastuzumab with pertuzumab, another antibody that binds a different epitope of HER2, preventing the formation of HER2-HER3 heterodimers, the most active forms in signaling[6]. The combination of this double anti-HER2 blockade with a taxane was approved by the FDA in 2012 for first-line treatment for HER2-positive advanced disease on the basis of results of the CLEOPATRA trial, which demonstrated an improvement in PFS and OS with this strategy[7].

Trastuzumab-emtansine (T-DM1): the first anti-HER2 ADC

Through continued research, another strategy was developed using a novel drug design technology for antibody-drug conjugates (ADCs), in which a potent cytotoxic agent is conjugated to an antibody with a linker to selectively deliver the payload to cells expressing a specific antigen, theoretically sparing normal cells from toxicity [Figure 1]. HER2-positive disease was an attractive target for ADC development. T-DM1, the first ADC, was designed to act against the HER2 receptor [Table 1]. The clinical benefit of T-DM1 in terms of both efficacy and toxicity was demonstrated in the EMILIA trial, which compared T-DM1 with the combination of capecitabine and the tyrosine kinase inhibitor (TKI) lapatinib. The results showed a significant improvement in PFS and OS with T-DM1, leading to the approval of T-DM1 by the FDA in 2013[8,9]. Later attempts to position T-DM1 in advanced first-line treatment or even in the neoadjuvant setting were not so successful, as there was no significant benefit in first-line treatment with T-DM1 compared with trastuzumab and a taxane or in the neoadjuvant early setting comparing TM-1 combined with pertuzumab and TCHP (docetaxel, carboplatin, trastuzumab, pertuzumab)[10,11]. However, in the KATHERINE trial, T-DM1 significantly decreased the risk of relapse in patients who had remaining residual disease after anti-HER2 neoadjuvant treatment[12].

A better understanding of the mechanisms of action of T-DM1 will help elucidate how primary and acquired resistance develop *in vivo*, and thus these mechanisms have been a subject of intense investigation[13]. The antitumor effects of T-DM1 reflect the activities of its components. Trastuzumab not

Table 1. T-DM1 main phase 3 trials

ADC	Characteristics	Trial/Population	Design	Results
Ado-trastuzumab emtansine T-DM1	Payload: DM1 DAR: 3,5 Linker: non-cleavable thioether Bystander effect: no	EMILIA[8,9]: HER2+ MBC previously treated taxane + trastuzumab	T-DM1 *vs.* Capecitabine + Lapatinib (C + L)	T-DM1 *vs.* C + L PFS: 9.6 m *vs.* 6.4 m. HR 0.68 (0.55-0.86) OS: 30.9 m *vs.* 25.1 m. HR 0.65 (0.55-0.77)
		TH3RESA: HER2+ MBC previously treated taxane, trastuzumab, lapatinib	T-DM1 *vs.* TPC	T-DM1 *vs.* TPC PFS: 6.2 m *vs.* 3.3 m. HR 0.53 (0.53-0.66) OS: 22.7 m *vs.* 15.8 m. HR 0.68 (0.54-0.85)
		MARIANNE[10]: HER2+ previously untreated MBC	Trastuzumab + taxane *vs.* T-DM1 *vs.* T-DM1 + pertuzumab	T-DM1 *vs.* Trastuzumab + taxane PFS: 14.1 m *vs.* 13.7 m. HR 0.91 (0.73-1.13) T-DM1 + pertuzumab *vs.* Trastuzumab + taxane PFS: 15.2 m *vs.* 13.7 m. HR 0.87 (0.69-1-08)
		KATHERINE[12]: HER2+ early disease. Residual invasive disease after neoadjuvant treatment with a taxane + trastuzumab	T-DM1 *vs.* Trastuzumab	TDM1 *vs.* Trastuzumab IDFS: 87.8% *vs.* 77.8%. HR 0.50 (0.39-0.64) OS: 94.35 *vs.* 92.5%. HR 0.70 (0.47-1.05)

ADC: Antibody-drug conjugate; DAR: drug-to-antibody ratio; MBC: metastatic breast cancer; m: month; HR: hazard ratio; PFS: progression-free survival; OS: overall survival; TPC: treatment of physician's choice; IDFS: invasive disease free survival.

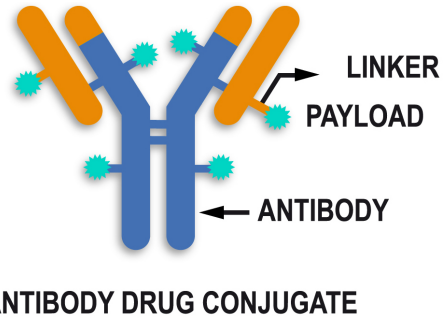

Figure 1. General structure of an antibody drug conjugate. The antibody drug conjugate contains three key components: antibody, linker, and payload.

only enables binding to tumor cells, but also inhibits HER2 signaling, HER2 extracellular domain shedding and ADCC. DM1 is a potent derivative of the maytansinoid toxin with a cytotoxic effect that is mediated through the inhibition of tubulin polymerization, which leads to the death of proliferating cells. Another key component is the non-cleavable thioether linker that conjugates trastuzumab and its payload, which allows the release of DM1 through liposomal degradation after the receptor-ADC complex has been internalized in the cell [Figure 2]. The drug-to-antibody ratio (DAR) for T-DM1 is 3.5:1 which defines the number of cytotoxic payloads held by each antibody. This is an important characteristic of an ADC that may be related to its potency[14]. Once the active payload lysine-MCC-DM1 complexes are released from the lysosome, cytotoxic effects are induced in the tumor cell but not in neighboring cells because of the membrane impermeability of the complexes[15]. Hence, there is no bystander effect from the killing of nearby tumor cells that do not present the antigen. Notably, loss or reduction of HER2 expression disables the internalization of ADC and thus represents the main mechanism of resistance[16,17]. Intratumor heterogeneity of HER2 expression leads to reduced access of T-DMI to non-HER2-expressing cells and

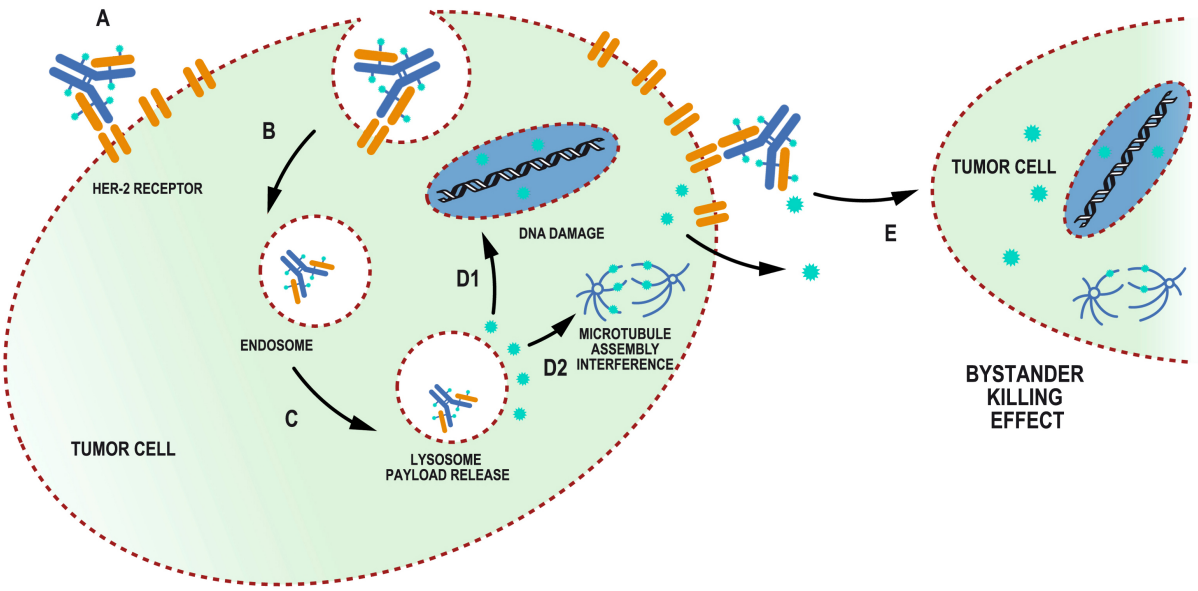

Figure 2. General mechanism of action of an antibody drug conjugate (ADC). A: The antibody binds to the HER2 receptor (RT) in the tumor cell and initiates the internalization process. B: The ADC-RT complex forms an endosome. C: Lysosomal degradation of the endosome releases the payload to the cytoplasm. D: The payload produces various cytotoxic effects, such as the following: D1, DNA damage, as with the topoisomerase I inhibitor in trastuzumab deruxtecan; D2, microtubule assembly interference, as with DM1 in trastuzumab emtansine; and E, bystander killing effect in neighbor tumor cells not expressing the antigen. After payload liberation in the tumor microenvironment by proteases, the payload, with cell membrane permeability, exhibits cytotoxic effects in neighboring tumor cells.

might influence primary resistance to T-DM1, as has been suggested in clinical trials[18,19]. Alterations in the internalization of the receptor-T-DM1, formation of endosomes and the lysosome pathway required for release of the DM1 payload have also been described in T-DM1 resistant cell lines in preclinical studies[13]. The upregulation of drug efflux transporters of which T-DM1 and other maytansinoids are substrates may also lead to resistance[20]. Other *in vitro* studies suggest that resistance to DM1-mediated cytotoxicity may result from the reduction of cyclins needed to promote cell progression to the mitotic phase, causing attenuation of mitotic catastrophe and apoptosis[21].

One of the strategies proposed to overcome or delay resistance to T-DM1 is the combination of T-DM1 with other drugs. The combination of T-DM1 with the anti-PD-L1 antibody atezolizumab was evaluated in the phase II trial KATE2 and showed improved PFS for the PD-L1-positive population but negative results for the intention-to-treat population[22]. The subsequent clinical trial, KATE3, is recruiting HER2+/PD-L1-positive patients with advanced breast cancer and will explore the efficacy of the combination. Furthermore, the Astefania study is examining the efficacy of the combination of T-DM1 and atezolizumab in patients with high-risk (N+) residual disease after neo-adjuvant chemo/trastuzumab/pertuzumab[23].

The combination of T-DM1 with anti-HER2 TKIs is another potential treatment strategy to improve efficacy and takes advantage of different anti-HER2 mechanisms of action as well as increasing activity in the brain since TKIs are able to cross the brain-blood-barrier. The HER2CLIMB 02 trial phase III is exploring the combination of T-DM1 with the reversible anti-HER2 TKI tucatinib[24].

NEW GENERATION OF ADCS

As another potential strategy to overcome resistance, a new generation of anti-HER2 ADCs, such as trastuzumab deruxtecan (T-DXd) and trastuzumab duocarmazine (SYD985), with an enhanced design that confers improved efficacy, is a current focus of research. There are new generation ADCs that are similar to T-DM1, as they use antibodies with the same sequence as trastuzumab, thus targeting the same antigen [Tables 2 and 3]. This is the case for trastuzumab deruxtecan and trastuzumab duocarmazine. Others, like ARX-788, use a different anti-HER2 antibody to bind to tumor cells. These ADCs also show differences in the linker technology design that permits them to be cleavable under specific conditions and do not require lysosomal degradation, as in T-DM1. The cytotoxic payloads have different mechanisms of action and properties, such as membrane permeability, which allows for killing of neighbor cells not presenting the antigen, enhancing the efficacy of these agents [Figure 2]. These properties also result in activity in tumors expressing lower levels of HER2 (defined as HER2-low tumors) and enable the treatment of HER2-negative patients that might also obtain clinical activity from these ADCs[25].

All these potential improvements are already being translated into a significant clinical benefit in HER2-positive metastatic breast cancer patients, as some of these new ADCs such as T-DXd and trastuzumab duocarmazine are already in an advanced phase of clinical development.

These promising therapies have raised interest in the scientific community, and several recent reviews describing the most updated data in this field have been published[26-28].

Trastuzumab-Deruxtecan

T-DXd is composed of trastuzumab linked by an enzymatically cleavable peptide-linker to DXd, which is an exatecan derivative, a potent topoisomerase I inhibitor that induces double-strand DNA breaks and apoptosis. The DAR of T-DXd is 7.7:1, which is higher than that of T-DM1 (3.5:1), allowing more delivery of payload by each ADC in target tumor cells. Preclinical results have shown promising antitumor effects in T-DM1-resistant cells and low HER2-expressing cells[29]. From these results, the initial clinical development plan targeted HER2-positive patients who had become resistant to T-DM1 or whose tumors were expressing low HER2 [defined as HER2 1+ and 2+ by immunochemistry with in situ hybridization (ISH) negative]. The phase I trial included 115 HER2-positive patients treated with the doses recommended for expansion that had received previous therapy (a median of seven lines); the overall response rate (ORR) was 60% in this heavily pretreated population. The toxicity profile showed more frequently gastrointestinal and hematological adverse events and 20 patients developed interstitial lung disease (ILD), including two treatment-related deaths from pneumonitis[30]. The phase 2 trial DESTINY-breast01 confirmed the clinical activity of T-DXd in HER2-positive patients resistant to T-DM1 and confirmed 5.4 mg/kg as the recommended dose for further development in phase III trials. A total of 184 patients received T-DXd at the recommended dose with a median of six previous lines of therapy in the metastatic setting. The primary endpoint was objective response following independent central review with other efficacy and safety secondary endpoints[31]. Updated results with a 20.5 median follow-up confirmed a 61.4% ORR and 19.4 months PFS with a median duration of response of 20.8 months. The most common G3 or higher adverse events were neutrophil count decrease, anemia, and nausea. Approximately 15.2% of cases had ILD, and 2.7% (5 patients) died[32]. As a result of the clinical activity demonstrated in this phase 2 trial for patients who had developed resistance to previous therapies including T-DM1, T-DXd was approved by the FDA and EMA for use in HER2-positive metastatic patients that had received at least two previous lines of treatment. The most relevant data on the clinical activity of T-DXd reported thus far is the head-to-head comparison with T-DM1 in the phase III DESTINY-breast03 trial. Patients with HER2-positive advanced breast cancer that had been previously treated with a taxane and trastuzuzmab were randomized at 1:1 to

Table 2. New generation anti-HER2 ADCs main trials in HER2-positive population with available results

ADC	Characteristics	Main trials in MBC	Design	Available results
Trastuzumab deruxtecan T-DXd	Payload: exatecan derivative DAR: 7,7 Linker: cleavable Bystander effect: yes	DESTINY-Breast 01[31,32] Phase 2: HER2+ MBC previously treated with T-DM1 NCT03248492	Single arm T-DXd	ORR: 61.4% PFS: 19.4 m (14.1-NE) OS: 24.6 m (23.1-NE)
		DESTINY-Breast 03[33] Phase 3: HER2+ MBC previously treated taxane + trastuzumab NCT03529110	T-DXd *vs.* T-DM1	T-DXd *vs.* T-DM1 ORR: 79.7% *vs.* 34.2% PFS: NR *vs.* 6.8 m. HR 0.28 (0.22-0.37) OS: NR *vs.* NR. HR 0.56 (0.36-0.86) 12 m OS: 94.1% *vs.* 85.9%.
Trastuzumab duocarmazine SYD985	Payload: vc-seco-DUBA DAR: 2.8 Linker: cleavable valine-citruline Bystander effect: yes	TULIP[41] Phase 3: HER2+ MBC previously treated with 2 lines or T-DM1 NCT03262935	SYD985 *vs.* TPC: Capecitabine + trastuzumab/lapatinib or Vinorelbine + trastuzumab or eribuline + trastuzumab	SYD985 *vs.* TPC PFS: 7 m *vs.* 4.9 m. HR 0.64 (0.49-0.84) OS: 20.4 m *vs.* 16.3 m. HR 0.83 (0.62-1.09)
ARX788	Payload: dolastatin monomethyl auristatin F Linker: non-cleavable AS269 DAR: 1.9 Bystander effect: no	ACE-Pan Tumor 01[43] Phase 1: advanced solid tumors with HER2 expression NCT03255070	ARX788	Breast cohort: PFS 17 m
RC48-ADC	Payload: monomethyl auristatin E DAR: 4 Linker: cleavable valine-citruline Bystander effect: no	Phase 1[45]: advanced breast cancer with HER2+ or HER2 low expression NCT03052634	RC48-ADC	Pooled results in HER2+ MBC: PFS: 4 to 6.3 m
ZW49	Payload: N-acyl sulfonamide auristatin DAR: 2 Linker: cleavable Bystander effect: NA	Phase 1I[51]: HER2 expressing cancers NCT03821233	ZW49	Results in HER2+ MBC: ORR: 13% PFS: NA

ADC: Antibody-drug conjugate; DAR: drug-to-antibody ratio; MBC: metastatic breast cancer; NA: non available; ORR: objective response rate; m: month; HR: hazard ratio; PFS: progression-free survival; OS: overall survival; TPC: treatment of physician's choice.

receive T-DXd or T-DM1. The primary endpoint was PFS and OS was a key secondary endpoint. The median follow-up for T-DXd was 16.2 months; the median PFS was 6.8 months for the T-DM1 groups, while the median PFS had not been reached for the T-DXd group, with a highly significant difference (HR of 0.28, 0.22-0.37, $P = 7.8 \times 10^{-22}$). The median OS had not been reached for both treatment arms, with a 12-month OS rate of 94.1% for the T-DXd group and 85.9% for the T-DM1 group, with no significant difference in this first analysis. The difference in ORR between the groups, with 79.7% for the T-DXd group compared with 34.2% for the T-DM1 group, was remarkable. The most common toxicity was hematological and gastrointestinal toxicity, with nausea as the most frequent event. Among the 524 randomized patients, 27 patients (10.5%) had ILD and 2 cases had G3 events with no cases of fatal pneumonitis for T-DXd compared with 5 cases of G1-2 for T-DM1[33]. These data currently position T-DXd as a standard second-line treatment in the metastatic setting, moving T-DM1 to later lines[34]. Another ongoing clinical trial is exploring the role of T-DXd in first-line treatment; this trial is comparing T-DXd combined with pertuzumab/placebo to the standard treatment of a taxane plus double blockade with trastuzumab and pertuzumab[35]. This ADC is likely to make its way to the early setting, because ongoing clinical trials are comparing it to adjuvant T-DM1 in post-neoadjuvant residual disease and another trial is exploring it as

Table 3. New generation anti-HER2 ADCs main trials in HER2-positive populationin progress

ADC	Characteristics	Main trials in MBC	Design	Available results
Trastuzumab deruxtecan T-DXd	Payload: exatecan derivative DAR: 7,7 Linker: cleavable Bystander effect: yes	DESTINY-Breast 02 Phase 3: HER2+ MBC previously treated with T-DM1 NCT03523585	T-DXd *vs.* TPC (Capecitabine + trastuzumab or lapatinib)	Results pending
		DESTINY-Breast 09 Phase 3: HER2+ previously untreated MBC NCT04784715	T-DXd + placebo *vs.* T-DXd + pertuzumab *vs.* Taxane + trastuzumab + pertuzumab	Recruiting
		DESTINY-Breast 05 Phase 3: HER2+ early disease. Residual invasive disease after neoadjuvant treatment with a taxane + trastuzumab NCT04622319	T-DXd *vs.* T-DM1	Recruiting
		DESTINY-Breast 11 Phase 3: HER2+ early disease neoadjuvant treatment NCT05113251	T-DXd *vs.* T-DXd - Taxane + trastuzumab + pertuzumab (THP) *vs.* dose dense AC - THP	Recruiting
Trastuzumab duocarmazine SYD985	Payload: vc-seco-DUBA DAR: 2.8 Linker: cleavable valine-citruline Bystander effect: yes	BYON5667.002 Phase I/II NCT04983238	SYD985+BYON5667(eye-drops)/placebo to reduce ocular toxicity	Results pending
ARX788	Payload: dolastatin monomethyl auristatin F Linker: non-cleavable AS269 DAR: 1.9 Bystander effect: no	ACE-Breast-03 Phase 2: HER2+ MBC resistant/refractory to T-DM1, and/or T-DXd, and/or Tucatinib NCT04829604	ARX788	Recruiting
RC48-ADC	Payload: monomethyl auristatin E DAR: 4 Linker: cleavable valine-citruline Bystander effect: no	Phase 2/3: HER2 + MBC with/without liver metastases NCT03500380	RC48-ADC *vs.* capecitabine + lapatinib	Recruiting
ZW49	Payload: N-acyl sulfonamide auristatin DAR: 2 Linker: cleavable Bystander effect: NA	Phase 1: HER2 expressing MBC NCT03821233	ZW49	Recruiting
MEDI4276	Payload: tubulysin-based microtubule inhibitor Linker: cleavable DAR: 4 Bystander effect: yes	Phase 1/2[41]: HER2 expressing breast or gastric/stomach cancers NCT02576548	MEDI4276	Completed

ADC: Antibody-drug conjugate; DAR: drug-to-antibody ratio; MBC: metastatic breast cancer; TPC: treatment of physician's choice; NA: non available.

initial neoadjuvant treatment alone or in sequence with paclitaxel, trastuzumab and pertuzumab (THP) compared with the sequential standard scheme of anthracycline-cyclophosphamide followed by THP[36,37]. Special attention and monitoring of potential T-DX-related ILD is being applied in these early disease trials, with clinical awareness and periodical imaging testing to rule out this potential severe toxicity.

Trastuzumab duocarmazine

Trastuzumab duocarmazine (SYD985) is another second-generation ADC that is composed of an antibody backbone with the same amino acidic sequence as trastuzumab with a cleavable linker to the vc-seco-DUBA payload, a potent duocarmacyn analog with a DAR of 2.8:1. The cytotoxic payload is an alkylant agent that

binds to the minor groove of DNA and exhibits activity in both dividing and non-dividing cells. The linker is cleaved by proteases of the lysosome after endocytosis and by proteases such as cathepsin B that are found extracellularly; thus, there is a bystander killer effect[38]. Preclinical studies that compared SYD985 with T-DM1 demonstrated encouraging activity of SYD985 in HER2-positive and HER2 low expression models, prompting exploration of SYD985 in a phase I trial in breast cancer and other HER2-expressing histologies[39]. In breast cancer dose-expansion cohorts, 33% of patients with HER2-positive breast cancer achieved objective partial responses; additionally, 28% of patients with HER2-low, hormone receptor-positive breast cancer and 40% with HER2-low, hormone receptor-negative breast cancer also showed objective partial response. The most common treatment-related adverse events were fatigue, conjunctivitis, and dry eye; most patients had at least one ocular adverse event[40]. The drug went directly into the TULIP trial, a phase III trial in a HER2-positive metastatic patient population that compared 2:1 SYD985 with the treatment of physician's choice (TPC) based on predefined standard options of a combination of chemotherapy and trastuzumab or lapatinib. Patients with two or more previous lines in the metastatic setting or with previous T-DM1 were included; patients had a median of four prior therapies. The primary endpoint of the study was centrally reviewed median PFS, which was 7.0 months for the SYD985 group and 4.9 months for the TPC group, with a statistically significant HR of 0.64 and no benefit in terms of OS in the first analysis. No significant differences were observed in ORR or health-related quality of life (HRQoL). The most frequently reported adverse events for SYD985 were conjunctivitis (38.2%), keratitis (38.2%) and fatigue (33.3%). Approximately 7.6% of patients treated with SYD985 showed ILD/pneumonitis, including two fatal events[41]. Ocular toxicity from this ADC may potentially be permanent, so it is crucial to treat it and find strategies to mitigate it.

ARX788

In addition to the two anti-HER2 ADCs described above, other ADCs are in the earlier phase of development, such as ARX788. This new generation ADC has a site-specific anti-HER2 antibody with an amino acid sequence different from that of trastuzumab; it uses a nonnatural amino acid-enabled conjugation technology and a non-cleavable Amberstatin (AS269) drug-linker, a highly potent tubulin inhibitor with a DAR of 1.9:1. Preclinical data demonstrated activity in HER2-high and HER2-low expression cell lines and xenograft and patient-derived xenograft models of breast and gastric cancer, motivating clinical development in these patient populations[42]. The phase I trial ACE-Breast-01 examined the use of ARX788 in HER2-positive metastatic breast cancer patients whose disease was resistant or refractory to HER2-targeted agents. The results showed a disease control of 100% and a median PFS of 17 months in the 29 patients treated at therapeutic doses who had been heavily pretreated in the advanced setting. However, there was some uncertainty about the anti-HER2 therapies to which this population would have been previously exposed. The safety profile was also favorable, with most adverse events being G1-G2; no dose-limiting toxicities were found and no drug-related deaths were reported. Since the cytotoxic payload of ARX788 is not a substrate of common efflux transporters, this is probably not a mechanism of resistance[43]. The phase 2 trial ACE-Breast-03 is ongoing and includes patients whose disease is resistant or refractory to T-DM1 and/or T-DXd and/or tucatinib-containing regimens[44].

RC48-ADC

RC48-ADC is a HER2-targeting ADC with a cleavable linker and a potent microtubule inhibitor payload MMAE with bystander killing effect in tumor cells. The DAR for this ADC is 4:1. Pooled results from two phase I studies in HER2-positive and HER2-low patients showed that among 70 HER2-positive patients, the ORR ranged from 22.2%-42.9% and the median PFS was 4-6.3 months for the different dosing levels. The most frequent G3 and above adverse events were decreased neutrophil count, increased GGT and fatigue[45]. This ADC is currently in various clinical trials including a phase II/III for HER2-positive patients that is comparing RC48-ADC with capecitabine and lapatinib[46]. As the field is moving forward, there are new

generation TKIs that are improving the results obtained with lapatinib, hence capecitabine and lapatinib might no longer be considered an optimal control arm in future clinical trials[47,48].

ZW49 and MEDI4276

Other new generation ADCs include zanidatamab zovodotin (ZW49), which contains a biparatopic antibody to optimize binding to target tumor cells. This ADC consists of the antibody ZW25 (zanidatamab) that binds to the same epitopes of the HER2 receptor as trastuzumab and pertuzumab and a cleavable linker to an aurastatine payload, with a DAR of 2:1. Preclinical data demonstrated antitumor activity in low and high HER2-expressing breast cancer cell lines and PDX models[49]. A phase I trial is currently ongoing in patients with locally advanced or metastatic HER2-expressing cancers; additional cohorts are being recruited[50]. Preliminary results have suggested promising efficacy in various types of HER2-positive tumors. In eight breast cancer patients with a median of six prior therapies treated at the cohort expansion recommended dose, the ORR was 13%. Toxicity analysis revealed two cases of G2 keratitis lasting more than 14 days; approximately 43% of patients exhibited keratitis, but all events decreased to G1 or eventually resolved. There were no ILD events or deaths related to treatment[51].

Another new generation ADC is MEDI4276, a biparatopic tetravalent antibody targeting two epitopes of the HER2 ecto-domain that has site-specific conjugation to a tubulysin-based microtubule inhibitor payload. Results from a phase I trial in advanced breast and gastric cancer demonstrated clinical activity, but also alteration of liver function tests and gastrointestinal toxicity[52].

ZW49 and MEDI4276 are representative examples of ADCs currently in clinical development for HER2-positive and HER2-low expressing populations, but there are many other ADCs. In the following years, new ADCs targeting HER2 will be developed. While ADCs are demonstrating unprecedented response rates and improvements in PFS that will likely translate in a clinically significant increase in OS, there is an important need to discover the mechanisms underlying the resistance that eventually develop as patients progress. A better understanding of the mechanisms underlying primary and acquired resistance will help inform the development of treatment strategies for patients. Knowledge of the presence of cross-resistance to payloads in different ADCs would help predict the absence of clinical benefit in a patient, thus avoiding unnecessary toxicity.

Combining ADCs

The combination of ADCs with other agents is a potential strategy to overcome or delay resistance and is being explored in clinical trials. The combination of ADCs with targeted agents is the most widely used approach, especially with TKIs and immune checkpoint inhibitors. Theoretically, these agents could enhance antitumor activity by targeting the intracellular domain of the HER2 receptor and trespassing the blood-brain-barrier (in the case of TKIs) or triggering innate and adaptive immunity (in the case of anti-PDL1 agents). Determining whether this combination strategy leads to improvement in efficacy without significantly increasing toxicity is critical.

Activity beyond HER2-positive: How low can we go

Research has demonstrated that a higher expression of HER2 corresponds with a greater clinical benefit. HER2 positivity has generally been defined by immunohistochemistry (IHC) and in situ hybridazation (ISH) and used to predict the clinical benefit of anti-HER2 therapies. Notably, the new generation ADCs are also demonstrating significant activity *in vitro* and clinical benefit in patients considered HER2-negative, redefining a so-called HER2-low population that would include tumors with IHC HER2 1+ and HER2 2+ with ISH negative. The threshold of HER2 expression from which a patient might benefit from these agents has not yet been clearly determined. Recent results from the phase II DAISY trial demonstrated activity of

T-DXd in heavily pretreated patients with different levels of HER2 expression. Notably, clinical activity was observed in the cohort that included patients with HER2-null tumors (IHC0+)[53]. These results suggest that the levels of HER2 expression needed for ADC to exert clinical activity might vary depending on the properties of the different linkers and payloads. The DAISY trial was designed to address the mechanisms of action and resistance to T-DXd. The results indicated that HER2 levels were significantly associated with efficacy, with a median PFS of 11 months for HER2-positive patients compared with 4 months for HER2-null patients. A high percentage and spatial distribution of HER2 IHC0+ cells was associated with a non-response to T-DXd. Approximately 65% of patients progressing to T-DXd had a decrease in HER2 expression compared with baseline levels[54]. There was a difference in the transcriptomic response to T-DXd depending on HER2 levels. No recurrent baseline driver mutations were identified as predictors of primary resistance, but 6% of patients presented an ERBB2 hemizygous deletion that might be associated with upfront resistance. SLX4 gene mutations were found in 20% of biopsies tested at progression, suggesting that SLX4 may be involved in a potential mechanism of acquired resistance[55].

Results from the phase 3 randomized study DESTINY-breast04 were reported in ASCO 2022 for the HER2-low population that accounts approximately for at least 50% of all metastatic breast cancer cases[25]. Patients with HR-positive tumors with one or two previous lines of therapy were included and randomized at 2:1 to receive T-DXd or TPC. There was a significant difference in PFS (the primary endpoint) of 10.1 months *vs.* 5.4 months, in favor of T-DXd, with a HR of 0.51. Furthermore, there was also a significant advantage in OS (23.9 months *vs.* 17.5 months) with a HR of 0.64. There was a similar benefit for the smaller exploratory subgroup of HR-negative patients included in the trial. These results led to FDA approval of T-DXd as the first ADC for treatment for a HER2-low population.

Brain metastases

As the survival of HER2-positive advanced cancer patients increases, the risk of the development of brain metastasis also increases. The activity of ADCs for patients with locally treated brain metastasis has been demonstrated in clinical trials for anti-HER2 ADCs, but there is not a wide representation of this population because of the restrictive inclusion criteria. Exploring how these agents perform in patients with non-treated or active brain disease is critical to design the best therapeutic sequence. Data from the phase IIIb single-arm KAMILLA clinical trial demonstrated the activity of T-DM1 in patients with brain metastases even in the absence of previous local treatment, challenging the hypothesis that larger molecules such as ADCs might not be capable of crossing a non-disrupted blood-brain barrier[56]. Preliminary data from DEBBRAH and TUXEDO trials with T-DXd were designed to address this question and demonstrated responses in patients with active brain metastases, both untreated patients and those progressing after previous local treatment[57,58]. The ongoing DESTINY-Breast12 trial is enrolling up to 250 patients with either active or stable brain metastases and should shed further light on the role of T-DXd in patients with CNS disease[59].

Mitigating toxicity

While current data on the potential effects of ADCs are encouraging, there is still room for improvement in terms of efficacy and toxicity. The toxicity profiles are different among ADCs depending on the payload used and the properties of the linkers that are cleavable under specific conditions. A better understanding of the toxicity profile of these agents is required to develop strategies to mitigate the most frequent adverse events along with adverse effects that are rare but might be severe. Identifying the mechanisms and risk factors that favor the occurrence of ILD is important to prevent ILD. A very strong research effort is ongoing, but so far, the most efficacious approach is an early diagnosis through radiological imaging and the identification of respiratory symptoms to enable treatment and initiate ILD management following available guidelines. There is also a need to understand the underlying causes of the eye toxicity that can be

found with these agents. In the case of SYD985, activation of the payload in the conjunctive tissue initially produces xerophthalmia, which may evolve into different ocular alterations including keratitis. One possible strategy for avoiding these ocular side effects is already under investigation in a clinical trial that is investigating SYD985 and eye drops specifically developed to inactivate the payload in the eye[60].

CONCLUSIONS

The new ADCs are rapidly changing the paradigm of treatment of HER2-positive advanced breast cancer patients and expanding the population that can benefit from them even in patients previously considered HER2-negative that have been defined as HER2-low.The results demonstrating the level of PFS achieved even in heavily pretreated populations indicate that these agents will improve survival. The proportion of HER2-positive metastatic patients that remain in a long-term response that could be considered potentially cured increases as new therapies are being developed, especially with the availability of these new generation ADCs. However, because a considerable number of patients will still eventually show disease progression, there is a need to continue developing more effective therapeutic options. A better understanding of the mechanisms of resistance to these agents is required to develop new strategies to overcome resistance as well as to define the best therapeutic sequence for each patient. Toxicity is still an issue and there is a need for better comprehension of the mechanisms and factors contributing to toxicity to develop mitigating strategies, especially with the implementation of these ADCs in the early stage disease as neo/adjuvant therapies.

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Cancer Drug Resistance

Original Article

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Multimodal 4-arylchromene derivatives with microtubule-destabilizing, anti-angiogenic, and MYB-inhibitory activities

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Abstract

Aim: Efficient and readily available anticancer drugs are sought as treatment options. For this reason, chromene derivatives were prepared using the one-pot reaction and tested for their anticancer and anti-angiogenic properties.

Methods: 2-Amino-3-cyano-4-(aryl)-7-methoxy-4H-chromene compounds (2A-R) were repurposed or newly synthesized via a three-component reaction of 3-methoxyphenol, various aryl aldehydes, and malononitrile. We performed assays to study the inhibition of tumor cell growth [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromid (MTT) assay], effects on microtubules (immunofluorescence), cell cycle (flow-activated cell sorting analysis), angiogenesis (zebrafish model), and MYB activity (luciferase reporter assay). Fluorescence microscopy was applied for localization studies via copper-catalyzed azide-alkyne click reaction of an alkyne-tagged drug derivative.

Results: Compounds 2A-C and 2F exhibited robust antiproliferative activities against several human cancer cell lines (50% inhibitory concentrations in the low nanomolar range) and showed potent MYB inhibition. The alkyne derivative 3 was localized in the cytoplasm after only 10 min of incubation. Substantial microtubule disruption and

G2/M cell-cycle arrest were observed, where compound 2F stood out as a promising microtubule-disrupting agent. The study of anti-angiogenic properties showed that 2A was the only candidate with a high potential to inhibit blood vessel formation *in vivo*.

Conclusion: The close interplay of various mechanisms, including cell-cycle arrest, MYB inhibition, and anti-angiogenic activity, led to identifying promising multimodal anticancer drug candidates.

Keywords: Chromene, pyran, anticancer drugs, microtubule, angiogenesis, MYB inhibition

INTRODUCTION

Multi-component reactions such as the Biginelli reaction, Van Leusen reaction, and the Ugi reaction, and their modifications are helpful for the design of biologically active compounds[1-4]. An efficient three-component synthesis of biologically active pyrans was reported using naphthol, phenol, and hydroxyquinoline derivatives, which led to several new anticancer compounds[5-8]. For instance, the naphthopyran LY290181 or 2-amino-4-(3-nitrophenyl)-4*H*-naphtho(1, 2-*b*)pyran-3-carbonitrile (1A) was identified as an active anticancer MDA (microtubule-binding/destabilizing agent)[9,10]. The close analog 1B was initially described as an apoptosis inducer in breast cancer and non-small-cell lung cancer cells[6]. It was also recently determined to be a highly potent inhibitor of the transcription factor MYB[11]. MYB is encoded by the proto-oncogene MYB and is involved in the development of malignancies, making it a potential therapeutic target[12]. The structurally optimized 7-methoxy-4*H*-chromene 2B is an even more potent apoptosis-inducing agent than 1B and showed high antiproliferative activities[5]. The 2-amino-4-aryl-5-oxo-4, 5-dihydropyrano[3,2-c]chromene-3-carbonitrile 1C inhibited centrosome clustering in melanoma cells, leading to the formation of multiple mitotic spindles, chromosome segregation defects, and cell death [Figure 1][13].

Based on these active anticancer pyran heterocycles, several 2-amino-3-cyano-4-aryl-7-methoxy-4*H*-chromenes were prepared for testing their antiproliferative and MYB-inhibitory activities. Experiments on cell death induction, microtubule destabilization, drug localization, and anti-angiogenic effects were performed to investigate the underlying mechanisms of action of the most active compounds.

METHODS

Chemistry

General

Starting compounds and reagents were obtained from Aldrich, TCI, and Alfa Aesar. The already published compounds 1A, 1B, 2B, 2O, and 2R were prepared as described[5,6,9,14]. For compound analysis, the following instruments were used: Gallenkamp for melting points (uncorrected); Perkin-Elmer Spectrum One FT-IR spectrophotometer (ATR) for IR spectra; BRUKER Avance 300 spectrometer for nuclear magnetic resonance spectra; chemical shifts were expressed as ppm (parts per million, δ) downfield from TMS (tetramethylsilane) as the internal standard; Varian MAT 311A (EI) and UPLC/Orbitrap (ESI-HRMS) for mass spectra; Perkin-Elmer 2400 CHN elemental analyzer for elemental analyses (microanalyses), and compounds were > 95% pure as to elemental analysis.

Synthesis

2-Amino-3-cyano-4-(3'-chloro-4', 5'-dimethoxyphenyl)-7-methoxy-4H-chromene (2A)

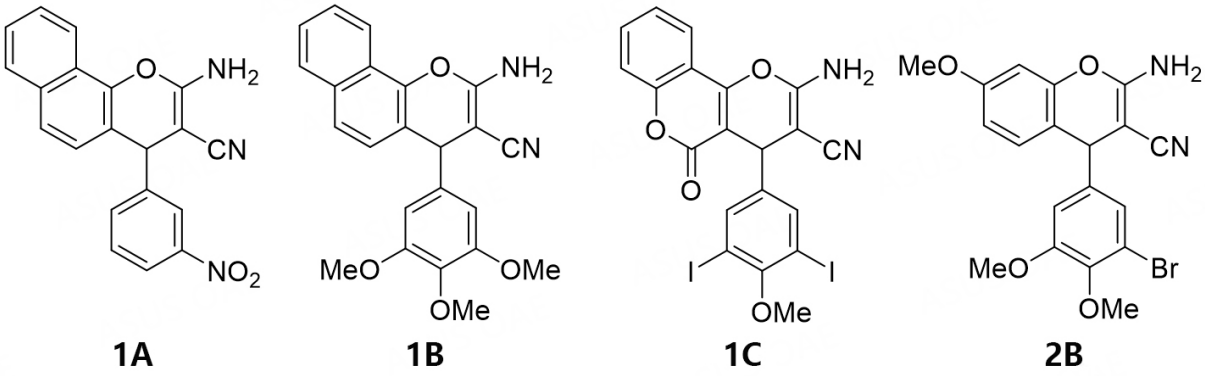


Figure 1. Structures of LY290181 (1A); MYB inhibitor Bcr-TMP (1B); chromosomal de-clustering agent 1C; and antiproliferative compound 2B.

3-Chloro-4, 5-dimethoxybenzaldehyde (200 mg, 1.0 mmol) and malononitrile (70 mg, 1.0 mmol) were dissolved in MeCN (5 mL), and three drops of Et_3N were added. The reaction mixture was stirred at room temperature for 30 min. 3-Methoxyphenol (109 µL, 1.0 mmol) was added, and the reaction mixture was heated with a heat gun and stirred at room temperature for 2 h. The precipitate was collected, washed with MeCN and *n*-hexane, and dried in a vacuum. Yield: 80 mg (0.22 mmol, 22%); colorless solid of mp = 224-225 °C; υ_{max}(ATR)/cm^{-1} 3397, 3332, 3220, 2943, 2846, 2186, 1651, 1619, 1572, 1509, 1465, 1430, 1415, 1399, 1313, 1281, 1258, 1231, 1207, 1193, 1178, 1148, 1137, 1124, 1051, 998, 928, 856, 834, 813, 801, 780, 753, 714, 690, 659; ^{1}H NMR (300 MHz, $CDCl_3$) 3.77 (3 H, s, OMe), 3.82 (6 H, s, 2 x OMe), 4.5-4.6 (3 H, m, 4-H, NH_2), 6.53 (1 H, d, J = 2.5 Hz, 8-H), 6.6-6.7 (3 H, m, 6'-H, 6-H), 6.71 (1 H, s, 2'-H), 6.85 (1 H, d, J = 8.7 Hz, 5-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 40.2 (4-C), 55.5 (OMe), 56.2 (OMe), 60.7 (OMe), 101.4 (8-C), 110.6 (6-C), 111.8 (6'-C), 113.9 (4a-C_q), 119.6 (CN), 121.2 (2'-C), 128.5 (3'-C), 130.1 (1'-C), 141.3 (4'-C), 149.1 (5'-C), 153.9 (8a-C_q), 159.1 (7-C), 159.6 (2-C); HRMS for $C_{19}H_{18}O_4N_2Cl$ [M^+ + H] calcd. 373.09496, found 373.09355.

2-Amino-3-cyano-4-(3'-iodo-4', 5'-dimethoxyphenyl)-7-methoxy-4H-chromene (2C)

Analogously to the synthesis of 2B, compound 2C (150 mg, 0.32 mmol, 32%) was obtained from 3-iodo-4, 5-dimethoxybenzaldehyde (292 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 223-224 °C; υ_{max}(ATR)/cm^{-1} 3403, 3334, 3220, 2937, 2841, 2186, 1651, 1619, 1582, 1563, 1508, 1479, 1464, 1411, 1397, 1308, 1272, 1253, 1231, 1200, 1178, 1136, 1124, 1044, 999, 875, 855, 812, 801, 777, 750, 688, 655, 611; ^{1}H NMR (300 MHz, dimethyl sulfoxide [DMSO]-d_6) 3.67 (3 H, s, OMe), 3.74 (3 H, s, OMe), 3.78 (3 H, s, OMe), 4.68 (1 H, s, 4-H), 6.56 (1 H, d, J = 2.5 Hz, 8-H), 6.68 (1 H, dd, J = 8.7 Hz, 2.5 Hz, 6-H), 6.9-7.1 (5 H, m, 6'-H, 6-H, 5-H, NH_2); ^{13}C NMR (75.5 MHz, DMSO-d_6) 39.1 (4-C), 55.4 (OMe), 55.6 (OMe), 55.9 (OMe), 93.0 (3'-C), 100.9 (8-C), 111.4 (6-C), 112.7 (6'-C), 114.8 (4a-C_q), 120.4 (CN), 128.2 (5-C), 129.9 (2'-C), 144.4 (1'-C), 146.9 (4'-C), 148.7 (5'-C), 152.2 (8a-C_q), 159.0 (7-C), 160.3 (2-C); *m/z* (%) 464 (43) [M^+], 201 (100); Anal. calcd. for $C_{19}H_{17}IN_2O_4$ (%), C, 49.16, H, 3.69, N, 6.03; found: C, 49.24, H, 3.77, N, 5.98.

2-Amino-3-cyano-4-(3', 5'-dichloro-4'-methoxyphenyl)-7-methoxy-4H-chromene (2D)

Analogously to the synthesis of 2B, compound 2D (115 mg, 0.31 mmol, 31%) was obtained from 3,5-dichloro-4-methoxybenzaldehyde (205 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 229 °C;

υ_{max}(ATR)/cm^{-1} 3395, 3331, 3219, 3004, 2973, 2938, 2841, 2192, 1652, 1619, 1583, 1557, 1509, 1478, 1448, 1404, 1395, 1318, 1301, 1282, 1265, 1254, 1195, 1150, 1127, 1082, 1043, 1029, 996, 949, 914, 888, 857, 848, 807, 795, 775, 751, 741, 687, 667, 653; ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6) 3.62 (3 H, s, OMe), 3.70 (3 H, s, OMe), 4.44 (1 H, s, 4-H), 5.58 (2 H, s, NH_2), 6.4-6.5 (2 H, m, 6-H, 8-H), 6.6-6.7 (1 H, m, 5-H), 6.96 (2 H, s, 2'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$/DMSO-d_6) 39.7 (4-C), 55.4 (OMe), 57.7 (3-C), 60.5 (OMe), 101.4 (8-C), 111.5 (6-C), 113.5 (4a-C_q), 120.2 (CN), 128.0 (2'-C, 6'-C), 129.3 (3'-C, 5'-C), 129.9 (5-C), 142.9 (1'-C), 149.0 (4'-C), 150.9 (8a-C_q), 159.5 (7-C), 160.0 (2-C); *m/z* (EI) 378 (16) [M$^+$], 376 (23) [M$^+$], 201 (100); Anal. calcd. for $C_{18}H_{14}Cl_2N_2O_3$ (%), C, 57.31, H, 3.74, N, 7.43; found: C, 57.40, H, 3.70, N, 7.36.

2-Amino-3-cyano-4-(3', 5'-dibromo-4'-methoxyphenyl)-7-methoxy-4H-chromene (2E)

Analogously to the synthesis of 2B, compound 2E (125 mg, 0.27 mmol, 31%) was obtained from 3, 5-dibromo-4-methoxybenzaldehyde (259 mg, 0.88 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 247 °C; υ_{max}(ATR)/cm^{-1} 3396, 3331, 3218, 2943, 2190, 1652, 1619, 1582, 1547, 1508, 1471, 1420, 1407, 1394, 1317, 1296, 1282, 1253, 1194, 1150, 1125, 1064, 1045, 1028, 999, 990, 947, 888, 857, 847, 811, 800, 779, 750, 733, 704, 686, 663; ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6) 3.70 (3 H, s, OMe), 3.76 (3 H, s, OMe), 4.52 (1 H, s, 4-H), 5.90 (2 H, s, NH_2), 6.47 (1 H, d, J = 2.5 Hz, 8-H), 6.55 (1 H, dd, J = 8.6 Hz, 2.5 Hz, 6-H), 6.77 (1 H, d, J = 8.6 Hz, 5-H), 7.23 (2 H, s, 2'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$/DMSO-d_6) 39.1 (4-C), 54.9 (OMe), 56.9 (3-C), 59.9 (OMe), 101.0 (8-H), 111.0 (6-C), 113.1 (4a-C_q), 117.6 (3'-C, 5'-C), 119.7 (CN), 129.4 (5-C), 131.3 (2'-C, 6'-C), 143.7 (1'-C), 148.6 (4'-C), 152.2 (8a-C_q), 159.0 (7-C), 159.7 (2-C); *m/z* (EI) 468 (8) [M$^+$], 466 (16) [M$^+$], 464 (8) [M$^+$], 201 (100); Anal. calcd. for $C_{18}H_{14}Br_2N_2O_3$ (%), C, 46.38, H, 3.03, N, 6.01; found: C, 46.29, H, 2.98, N, 6.06.

2-Amino-3-cyano-4-(3, 5-diiodo-4-methoxyphenyl)-7-methoxy-4H-chromene (2F)

Analogously to the synthesis of 2B, compound 2F (165 mg, 0.30 mmol, 30%) was obtained from 3, 5-diiodo-4-methoxybenzaldehyde (388 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 228 °C; υ_{max}(ATR)/cm^{-1} 3400, 3332, 3219, 2936, 2841, 2187, 1652, 1619, 1580, 1534, 1506, 1460, 1405, 1387, 1316, 1293, 1281, 1247, 1198, 1150, 1124, 1048, 1028, 993, 945, 897, 885, 854, 808, 798, 778, 749, 737, 711, 708, 700, 685; ^{1}H NMR (300 MHz, $CDCl_3$/DMSO-d_6) 3.70 (3 H, s, OMe), 3.72 (3 H, s, OMe), 4.49 (1 H, s, 4-H), 6.07 (2 H, s, NH_2), 6.47 (1 H, d, J = 2.6 Hz, 8-H), 6.54 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.77 (1 H, d, J = 8.6 Hz, 5-H), 7.47 (2 H, s, 2'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$/DMSO-d_6) 54.9 (OMe), 56.6 (3-C), 59.9 (OMe), 90.3 (2'-C, 6'-C), 100.9 (8-C), 111.0 (6-C), 113.3 (4a-C_q), 119.8 (CN), 129.4 (5-C), 138.4 (2'-C, 6'-C), 144.9 (1'-C), 148.6 (8a-C_q), 156.9 (4'-C), 158.9 (7-C), 159.7 (2-C); *m/z* (EI) 560 (52) [M$^+$], 201 (100); Anal. calcd. for $C_{18}H_{14}I_2N_2O_3$ (%), C, 38..60, H, 2.52, N, 5.00; found: C, 38.69, H, 2.45, N, 4.97.

2-Amino-3-cyano-4-(2'-fluorophenyl)-7-methoxy-4H-chromene (2G)

Analogously to the synthesis of 2B, compound 2G (73 mg, 0.25 mmol, 25%) was obtained from 2-fluorobenzaldehyde (124 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Yield: 73 mg (0.25 mmol, 25%); colorless solid of m.p. 198 °C; υ_{max}(ATR)/cm^{-1} 3405, 3336, 3214, 2194, 1652, 1619, 1582, 1509, 1486, 1453, 1435, 1405, 1317, 1292, 1255, 1240, 1224, 1193, 1152, 1124, 1103, 1092, 1045, 1031, 937, 871, 857, 817, 803, 787, 753, 721, 705, 679; ^{1}H NMR (300 MHz, $CDCl_3$) 3.75 (3 H, s, OMe), 4.65 (2 H, s, NH_2), 5.04 (1 H, s, 4-H), 6.51 (1 H, d, J = 2.6 Hz, 8-H), 6.59 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.92 (1 H, d, J = 8.6 Hz, 5-H), 7.0-7.2 (4 H, m, 3'-H, 4'-H, 5'-H,

6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 33.8 (4-C), 55.5 (OMe), 59.2 (3-C), 101.3 (8-C), 111.7 (6-C), 114.1 (4a-C_q), 115.8 (d, J = 22.0 Hz, 3'-C), 119.6 (CN), 124.6 (5'-C), 128.9 (d = 8.3 Hz, 4'-C), 129.7 (5-C), 131.5 (d = 12.7 Hz, 6'-C), 149.2 (8a-C_q), 159.4 (7-C), 159.8 (2-C), 160.0 (d = 195.9 Hz, 2'-C); *m/z* (EI) 296 (42) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{13}FN_2O_2$ (%), C, 68.91, H, 4.42, N, 9.45; found: C, 68.99, H, 4.36, N, 9.39.

2-Amino-3-cyano-4-(2'-chlorophenyl)-7-methoxy-4H-chromene (2H)

Analogously to the synthesis of 2B, compound 2H (500 mg, 1.6 mmol, 40%) was obtained from 2-chlorobenzaldehyde (562 mg, 4.0 mmol), malononitrile (280 mg, 4.0 mmol), six drops of Et_3N, and 3-methoxyphenol (436 µL, 4.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 184 °C; υ_{max}(ATR)/cm^{-1} 3435, 3343, 3216, 2937, 2840, 2186, 1639, 1618, 1580, 1506, 1466, 1441, 1402, 1311, 1291, 1278, 1253, 1198, 1154, 1123, 1033, 956, 935, 874, 854, 817, 782, 758, 724, 708, 694, 675; ^{1}H NMR (300 MHz, $CDCl_3$) 3.75 (3 H, s, OMe), 4.62 (2 H, s, NH_2), 5.31 (1 H, s, 4-H), 6.51 (1 H, d, J = 2.6 Hz, 8-H), 6.57 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.89 (1 H, d, J = 8.6 Hz, 5-H), 7.2-7.3 (3 H, m, 4'-H, 5'-H, 6'-H), 7.3-7.4 (1 H, m, 3'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 36.8 (3-C), 55.5 (OMe), 59.7 (4-C), 101.3 (8-H), 111.6 (6-C), 114.2 (4a-C_q), 119.5 (CN), 127.5 (6'-C), 128.4 (5'-C), 129.7 (4'-C), 129.9 (3'-C), 130.7 (5-C), 133.0 (2'-C), 141.8 (1'-C), 149.1 (8a-C_q), 159.5 (7-C), 159.6 (2-C); *m/z* (EI) 314 (6) [M^+], 312 (24) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{13}ClN_2O_2$ (%), C, 65.29, H, 4.19, N, 8.96; found: C, 65.36, H, 4.12, N, 9.02.

2-Amino-3-cyano-4-(3', 4'-difluorophenyl)-7-methoxy-4H-chromene (2I)

Analogously to the synthesis of 2B, compound 2I (98 mg, 0.31 mmol, 31%) was obtained from 3, 4-difluorobenzaldehyde (142 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol) three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Yield: 98 mg (0.31 mmol, 31%); colorless solid of m.p. 193 °C; υ_{max}(ATR)/cm^{-1} 3433, 3349, 3220, 2842, 2186, 1663, 1616, 1580, 1507, 1464, 1436, 1408, 1311, 1289, 1255, 1202, 1191, 1156, 1123, 1109, 1046, 959, 928, 889, 856, 828, 816, 802, 775, 746, 697, 673; ^{1}H NMR (300 MHz, $CDCl_3$) 3.77 (3 H, s, OMe), 4.61 (2 H, s, NH_2), 4.64 (1 H, s, 4-H), 6.53 (1 H, d, J = 2.6 Hz, 8-H), 6.62 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.81 (1 H, d, J = 8.6 Hz, 5-H), 6.9-7.0 (2 H, m, 5'-H, 6'-H), 7.0-7.1 (1 H, m, 2'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 39.8 (4-C), 55.5 (OMe), 60.5 (3-C), 101.5 (8-C), 111.9 (6-C), 113.8 (4a-C_q), 116.7 (d, J = 17.6 Hz, 2'-C), 117.4 (d, J = 17.4 Hz, 5'-C), 119.4 (CN), 123.7 (d, J = 9.8 Hz, 6'-C), 130.1 (5-C), 141.8 (1'-C), 147.1 (d, J = 156.0 Hz), 149.1 (8a-C_q), 151.2 (d, J = 156.0 Hz, 3'-C), 159.1 (7-C), 159.7 (2-C); *m/z* (EI) 314 (33) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{12}F_2N_2O_2$ (%), C, 64.97, H, 3.85, N, 8.91; found: C, 65.08, H, 3.79, N, 8.96.

2-Amino-3-cyano-4-(3'-chloro-4'-fluorophenyl)-7-methoxy-4H-chromene (2J)

Analogously to the synthesis of 2B, compound 2J (100 mg, 0.30 mmol, 30%) was obtained from 3-chloro-4-fluorobenzaldehyde (159 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 192-193 °C. υ_{max}(ATR)/cm^{-1} 3432, 3350, 3285, 3219, 3087, 2972, 2842, 2186, 1659, 1615, 1579, 1505, 1495, 1464, 1435, 1412, 1401, 1320, 1306, 1291, 1254, 1221, 1201, 1153, 1122, 1058, 1044, 948, 897, 855, 828, 815, 801, 774, 755, 711, 702, 693; ^{1}H NMR (300 MHz, $CDCl_3$) 3.77 (3 H, s, OMe), 4.61 (2 H, s, NH_2), 4.64 (1 H, s, 4-H), 6.54 (1 H, d, J = 2.6 Hz, 8-H), 6.61 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.80 (1 H, d, J = 8.6 Hz, 5-H), 7.0-7.1 (2 H, m, 5'-H, 6'-H), 7.1-7.2 (1 H, m, 2'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 39.7 (4-C), 55.6 (OMe), 60.5 (3-C), 101.5 (8-C), 112.0 (6-C), 113.8 (4a-C_q), 116.8 (d, J = 21.3 Hz, 5'-C), 119.4 (CN), 121.4 (d, J = 18.0 Hz, 3'-C), 127.6 (6'-C), 130.0 (2'-C), 131.1 (5-C), 141.9 (1'-C), 149.1 (8a-C_q), 157.3 (d, J = 248.9 Hz, 4'-C), 159.1 (7-C), 159.7 (2-C); *m/z* (%) 330 (47) [M^+], 201 (100), 186 (43), 158 (43). Anal. calcd. for $C_{17}H_{12}ClFN_2O_2$ (%), C, 61.74, H, 3.66, N, 8.47; found: C, 61.84, H, 3.71, N, 8.50.

2-Amino-3-cyano-4-(3', 4'-dichlorophenyl)-7-methoxy-4H-chromene (2K)

Analogously to the synthesis of 2B, compound 2K (105 mg, 0.30 mmol, 30%) was obtained from 3, 4-dichlorobenzaldehyde (175 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 192 °C; υ_{max}(ATR)/cm^{-1} 3433, 3349, 3220, 2186, 1661, 1616, 1579, 1506, 1463, 1434, 1412, 1320, 1290, 1251, 1199, 1180, 1157, 1123, 1046, 1029, 948, 911, 895, 885, 856, 825, 809, 786, 753, 732, 703, 692, 665; ^{1}H NMR (300 MHz, $CDCl_3$) 3.77 (3 H, s, OMe), 4.63 (1 H, s, 4-H), 4.66 (2 H, s, NH_2), 6.53 (1 H, d, J = 2.6 Hz, 8-H), 6.61 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.80 (1 H, d, J = 8.6 Hz, 5-H), 7.03 (1 H, dd, J = 8.2 Hz, 2.1 Hz, 6'-H), 7.22 (1 H, d, J = 2.1 Hz, 2'-H), 7.37 (1 H, d, J = 8.2 Hz, 5'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 39.8 (4-C), 55.5 (OMe), 60.0 (3-C), 101.5 (8-C), 111.9 (6-C), 113.5 (4a-C_q), 119.4 (CN), 127.3 (6'-C), 129.8 (2'-C), 130.1 (5-C), 130.8 (5'-C), 131.4 (4'-C), 132.9 (3'-C), 145.0 (1'-C), 149.0 (8a-C_q), 159.2 (7-C), 159.7 (2-C); *m/z* (EI) 348 (7) [M^+], 346 (13) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{12}Cl_2N_2O_2$ (%), C, 58.81, H, 3.48, N, 8.07; found: C, 58.94, H, 3.55, N, 8.09.

2-Amino-3-cyano-4-(2', 4'-difluorophenyl)-7-methoxy-4H-chromene (2L)

Analogously to the synthesis of 2B, compound 2L (100 mg, 0.32 mmol, 32%) was obtained from 2, 4-difluorobenzaldehyde (142 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 169-170 °C. υ_{max}(ATR)/cm^{-1} 3432, 3349, 3220, 3084, 3015, 2969, 2936, 2840, 2186, 1660, 1615, 1602, 1581, 1497, 1463, 1409, 1316, 1280, 1262, 1241, 1220, 1202, 1190, 1155, 1138, 1124, 1086, 1039, 960, 852, 827, 808, 781, 751, 730, 683; ^{1}H NMR (300 MHz, $CDCl_3$) 3.76 (3 H, s, OMe), 4.63 (2 H, s, NH_2), 5.01 (1 H, s, 4-H), 6.52 (1 H, d, J = 2.6 Hz, 8-H), 6.60 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.7-6.8 (2 H, m, 3'-H, 5'-H), 6.88 (1 H, d, J = 8.6 Hz, 5-H), 7.0-7.1 (1 H, m, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 33.5 (4-C), 55.5 (OMe), 59.2 (3-C), 101.4 (8-C), 103.8-104.5 (m, 3'-C), 111.6-112.0 (m, 5'-C, 6-C), 113.8 (4a-C_q), 119.4 (CN), 127.5 (d, J = 12.9 Hz), 129.7 (5-C), 130.5-130.7 (m, 6'-C), 139.0 (1'-C), 149.2 (8a-C_q), 159.6 (7-C), 159.8 (2-C), 160.3 (d, J = 247.5 Hz, 4'-C), 162.0 (d, J = 248.8 Hz, 2'-C); *m/z* (%) 314 (43) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{12}F_2N_2O_2$ (%), C, 64.97, H, 3.85, N, 8.91; found: C, 64.90, H, 3.78, N, 8.88.

2-Amino-3-cyano-4-(2', 3'-difluorophenyl)-7-methoxy-4H-chromene (2M)

Analogously to the synthesis of 2B, compound 2M (100 mg, 0.32 mmol, 32%) was obtained from 2, 3-difluorobenzaldehyde (142 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 183-184 °C. υ_{max}(ATR)/cm^{-1} 3429, 3331, 3213, 2975, 2935, 2840, 2193, 1652, 1627, 1614, 1576, 1508, 1478, 1416, 1310, 1287, 1266, 1249, 1231, 1190, 1150, 1123, 1043, 1030, 969, 929, 895, 857, 824, 792, 782, 758, 740, 718, 697, 683; ^{1}H NMR (300 MHz, $CDCl_3$) 3.76 (3 H, s, OMe), 4.64 (2 H, s, NH_2), 5.05 (1 H, s, 4-H), 6.52 (1 H, d, J = 2.6 Hz, 8-H), 6.60 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.9-7.1 (4 H, m, 5-H, 4'-H, 5'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 34.1 (4-C), 55.5 (OMe), 58.9 (3-C), 101.5 (8-C), 111.8 (6-C), 113.5 (4a-C_q), 116.1 (d, J = 17.2 Hz, 4'-C), 119.3 (CN), 124.3-124.5 (m, 6'-C), 129.7 (5-C), 133.9 (d, J = 9.8 Hz, 5'-C), 148.1 (d, J = 151.0 Hz, (2'-C), 149.2 (8a-C_q), 151.4 (d, J = 156.0 Hz, 3'-C), 159.7 (7-C), 159.9 (2-C); *m/z* (%) 314 (27) [M^+], 201 (100); Anal. calcd. for $C_{17}H_{12}F_2N_2O_2$ (%), C, 64.97, H, 3.85, N, 8.91; found: C, 64.88, H, 3.80, N, 8.94.

2-Amino-3-cyano-4-(2', 5'-difluorophenyl)-7-methoxy-4H-chromene (2N)

Analogously to the synthesis of 2B, compound 2N (95 mg, 0.30 mmol, 30%) was obtained from 2, 5-difluorobenzaldehyde (142 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol), three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 179-180°C. υ_{max}(ATR)/cm^{-1} 3629, 3458, 3408, 3333, 3213, 3080, 2983, 2938, 2843, 2192, 1651, 1618, 1579, 1508, 1490, 1452, 1436, 1404, 1317, 1290, 1252, 1240, 1190, 1176, 1150, 1122, 1082, 1044, 1028, 961, 942, 883, 855, 849, 817, 806, 794, 777, 752, 739, 731, 716, 700; ^{1}H NMR (300 MHz, $CDCl_3$) 3.76 (3 H, s, OMe), 4.67 (2 H, s, NH_2), 5.04 (1 H, s, 4-H), 6.52 (1 H, d, J = 2.6 Hz, 8-H), 6.61 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.8-7.0 (4 H, m, 5-H, 3'-H, 4'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 33.8 (4-C), 55.5 (OMe), 58.8 (3-C), 101.5 (8-C), 111.9 (6-C), 113.4 (4a-C_q), 115.2-116.2 (m, 4'-C), 116.7-117.2 (m, 3'-C, 6'-C), 119.3 (CN), 129.7 (5-C), 133.1-133.4 (m, 1'-C), 149.2 (8a-C_q), 156.2 (d, J = 239.8 Hz, 2'-C), 159.0 (d, J = 243.3 Hz, 5'-C), 159.7 (7-C), 159.9 (2-C); *m/ z* (%) 314 (60) [M^+], 201 (100), 186 (37), 158 (33); Anal. calcd. for $C_{17}H_{12}F_2N_2O_2$ (%), C, 64.97, H, 3.85, N, 8.91; found: C, 65.04, H, 3.90, N, 8.98.

2-Amino-3-cyano-4-(3', 4', 5'-trifluorophenyl)-7-methoxy-4H-chromene (2P)

Analogously to the synthesis of 2B, compound 2P (105 mg, 0.32 mmol, 32%) was obtained from 3, 4, 5-trifluorobenzaldehyde (160 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol) three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 237-238 °C; υ_{max}(ATR)/cm^{-1} 3428, 3346, 3221, 3053, 3014, 2945, 2848, 2187, 1662, 1616, 1579, 1524, 1506, 1465, 1446, 1409, 1342, 1310, 1292, 1232, 1203, 1182, 1156, 1131, 1116, 1035, 985, 897, 879, 855, 834, 822, 803, 785, 756, 716, 709, 688; ^{1}H NMR (300 MHz, DMSO-d_6) 3.79 (3 H, s, OMe), 4.81 (1 H, s, 4-H), 6.57 (1 H, d. J = 2.5 Hz, 8-H), 6.69 (1 H, dd, J = 8.7 Hz, 2.5 Hz, 6-H), 6.98 (1 H, d, J = 8.7 Hz, 5-H), 7.07 (2 H, s, NH_2), 7.1-7.2 (2 H, m, 2'-H, 6'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$/DMSO-d_6) 54.8 (3-C), 55.4 (OMe), 101.1 (8-C), 111.5-112.0 (m, 2'-C, 6'-C, 6-C), 113.8 (4a-C_q), 120.2 (CN), 129.9 (5-C), 136.0 (1'C), 143.3 (4'-C), 148.8 (8a-C_q), 150.2 (dd, J = 248.7 Hz, 9.8 Hz, 3'-C, 5'-C), 159.2 (7-C), 160.4 (2-C); HR-MS (ESI, *m/z*) for $C_{17}H_{12}O_2N_2F_3$ [M^+ + H] calcd. 333.08454, found 333.08436.

2-Amino-3-cyano-4-(3'-pentafluorothiophenyl)-7-methoxy-4H-chromene (2Q)

Analogously to the synthesis of 2B, compound 2Q (130 mg, 0.32 mmol, 32%) was obtained from 3-pentafluorothiobenzaldehyde (232 mg, 1.0 mmol), malononitrile (70 mg, 1.0 mmol) three drops of Et_3N, and 3-methoxyphenol (109 µL, 1.0 mmol) in MeCN (5 mL). Colorless solid of m.p. 226-227 °C; υ_{max}(ATR)/cm^{-1} 3472, 3321, 3281, 3233, 3205, 3071, 2936, 2841, 2192, 1652, 1626, 1612, 1577, 1508, 1464, 1434, 1402, 1293, 1252, 1191, 1155, 1125, 1111, 1097, 1036, 938, 911, 884, 833, 810, 794, 780, 747, 717, 697, 688; ^{1}H NMR (300 MHz, $CDCl_3$) 3.77 (3 H, s, OMe), 4.64 (2 H, s, NH_2), 4.75 (1 H, s, 4-H), 6.55 (1 H, d, J = 2.6 Hz, 8-H), 6.62 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.80 (1 H, d, J = 8.6 Hz, 5-H), 7.3-7.4 (2 H, m, 5'-H, 6'-H), 7.53 (1 H, s, 2'-H), 7.6-7.7 (1 H, m, 4'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$) 40.5 (4-C), 55.5 (OMe), 60.2 (3-C), 101.6 (8-C), 112.0 (6-C), 113.5 (4a-C_q), 119.3 (CN), 124.9 (2'-C), 125.3 (4'-C), 129.3 (5'-C), 130.1 (5-C), 131.1 (6'-C), 145.9 (1'-C), 149.2 (8a-C_q), 154.3-155.0 (m, 3'-C), 159.3 (7-C), 159.8 (2-C); *m/z* (%) 404 (31) [M^+], 201 (100), 186 (38), 158 (33); Anal. calcd. for $C_{17}H_{12}F_5N_2O_2S$ (%), C, 50.50, H, 3.24, N, 6.93; found: C, 50.59, H, 3.20, N, 6.88.

2-Amino-3-cyano-4-(3'-bromo-4', 5'-dimethoxyphenyl)-7-propargyloxy-4H-chromene (3)

A mixture of malononitrile (70 mg, 1.0 mmol) and 3-bromo-4, 5-dimethoxybenzaldehyde (245 mg, 1.0 mmol) in ethanol (EtOH) (5 mL) was treated with three drops of piperidine, and the reaction mixture was stirred for 30 min at room temperature. 3-Propargyloxyphenol (148 mg, 1.0 mmol) was added, and the

reaction mixture was stirred under reflux for 4 h. The solvent was evaporated, and the residue was taken up in ethyl acetate, washed with water, and dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by column chromatography (silica gel 60). Yield: 50 mg (0.11 mmol, 11%); off-white solid of m.p. 206-207 °C; R_f = 0.47 (ethyl acetate / *n*-hexane, 1:1); υ_{max}(ATR)/cm^{-1} 3442, 3331, 3294, 3240, 3202, 3009, 2971, 2939, 2834, 2192, 1651, 1629, 1608, 1570, 1508, 1488, 1459, 1431, 1401, 1312, 1284, 1230, 1183, 1157, 1124, 1044, 1028, 995, 880, 850, 840, 823, 801, 780, 762, 684, 654; ^{1}H NMR (300 MHz, DMSO-d_6) 3.59 (1 H, s, CCH), 3.70 (3 H, s, OMe), 3.81 (3 H, s, OMe), 4.72 (1 H, s, 4-H), 4.80 (2 H, s, CH_2), 6.65 (1 H, d, J = 2.6 Hz, 8-H), 6.73 (1 H, dd, J = 8.6 Hz, 2.6 Hz, 6-H), 6.88 (1 H, s, 6'-H), 7.0-7.1 (4 H, m, 5-H, 2'-H, NH_2); ^{13}C NMR (75.5 MHz, DMSO-d_6) 55.4 (CH_2), 55.7 (3-C), 56.1 (OMe), 60.0 (OMe), 78.6 (C*CH*), 78.9 (*C*CH), 102.5 (8-C), 111.8 (6-C), 112.3 (4a-C_q), 115.4 (6'-C), 116.8 (3'-C), 120.4 (CN), 122.5 (2'-C), 129.9 (5-C), 143.6 (1'-C), 144.4 (4'-C), 148.6 (5'-C), 153.4 (8a-C_q), 156.9 (7-C), 160.4 (2-C); HR-MS (ESI, *m/z*) for $C_{21}H_{18}O_4N_2{}^{79}Br$ [M^+ + H] calcd. 441.04445, found 441.04419.

Biological assays

Cell lines and culture conditions

The following cell lines were used: 518A2 melanoma (Department of Radiotherapy, Medical University of Vienna, Austria)[15], HeLa cervix carcinoma, KBV1^{Vbl} (ACC-149 cervix carcinoma, MCF7 [ACC-115]) breast carcinoma, U-87 glioblastoma, HT-29 (ACC-299), HCT-116 (ACC-581) and HCT-116p53-/- colon carcinoma, EA.hy926 (ATCC® CRL-2922™) endothelial hybrid cells, and HDFa (ATCC® PCS-201-012™) adult human dermal fibroblasts. The cells were maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (20% for HDFa cells) and 1% ZellShield® at 37 °C, 5% CO_2, and 95% humidity. KB-V1^{Vbl} cells were treated with 340 nM vinblastine to retain vinblastine resistance. Only mycoplasma-free cultures were used.

In vitro cytotoxicity assay

An 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromid (MTT) assay was used for antiproliferative studies. Cancer cells (5 × 10^4 cells/mL, 100 µL/well) were placed in 96-well plates (10 × 10^4 cells/mL for HDFA and U87 cells), and the cells were incubated for 24 h at 5% CO_2 and 95% humidity. Then, cells were treated with test compounds (at various concentrations) or solely with DMSO as a negative control for 72 h. Then, 12.5 µL of a 0.5% MTT solution in phosphate-buffered saline (PBS) was added to the cells, followed by incubation for 2 h at 37 °C. After centrifugation of the plates (300 × g, 5 min, 4 °C), the medium was discarded, and 25 µL of DMSO containing 10% SDS and 0.6% acetic acid was added, followed by incubation at 37 °C for at least 1 h. Formazan absorbance at λ = 570 nm was measured using a Tecan infinite F200 microplate reader and corrected for the background (λ = 630 nm). The 50% inhibitory concentration (IC_{50}) values were calculated from dose-response curves (means ± SD, four independent experiments) compared to DMSO-treated control cells, which were set to 100%. GraphPad Prism 9 was used for curve-fitting.

Microtubule immunofluorescence staining

518A2 melanoma cells (10 × 10^4 cells/mL, 0.5 mL/well) were placed on coverslips in 24-well plates followed by incubation at 37 °C, 5% CO_2, and 95% humidity. The cells were treated with test compounds and controls (25 and 100 nm) for 0.5, 1, 3, and 6 h and washed with cytoskeletal buffer (100 nm PIPES, 3 nm $MgCl_2$, 138 nm KCl, 2 nm EGTA, 300 nm sucrose, pH 6.8). Fixation and permeabilization were performed for 5 min with 3.7% formaldehyde and 0.2% Triton X-100 in a cytoskeletal buffer. The cells were fixed with cold EtOH for 10 s, rehydrated in PBS, and blocked with 1% bovine serum albumin in PBS for 30 min. The microtubules were stained for 1 h with primary (anti-α-tubulin, mouse monoclonal antibody) and secondary antibodies (goat anti-mouse IgG-AF-546, Invitrogen) and washed with PBS between each treatment. Nuclei were stained using DAPI (1 µg/mL in PBS) for 30 min. The coverslips were placed in

Roti®-Mount FluorCare. Microtubules (λ_{ex} = 488 nm, λ_{em} = 507 nm) and nuclei (λ_{ex} = 358 nm, λ_{em} = 461 nm) were documented by confocal microscopy (Leica TCS SP5 confocal microscope, 630 × magnification) and edited with *ImageJ*.

Intracellular localization

518A2 melanoma cells (7.5 × 10^4 cells/mL, 0.5 mL/well) were placed on coverslips in 24-well plates and cultivated for 24 h. Cells were treated with **3** (25 µm, 0.4% tween 80 in PBS) for 10 min at room temperature. After fixation in 3.7 % formaldehyde for 15 min and washing with PBS, the cells were incubated with the "click-solution" (2 mm $CuSO_4$, 5 mm sodium ascorbate, 0.1 mm 3-azido-7-hydroxycoumarin, 1% bovine serum albumin in PBS) for 30 min and washed with PBS. The nuclei were counterstained with Nuclear Green LCS1 for another 30 min, and the coverslips were mounted in Roti®-Mount FluorCare. The clicked test compound (λ_{ex} = 404 nm, λ_{em} = 477 nm) and nuclei (λ_{ex} = 488 nm, λ_{em} = 507 nm) were analyzed with a Leica TCS SP5 confocal microscope, and the obtained images were edited with *ImageJ*.

Cell-cycle analysis

518A2 melanoma cells (10 × 10^4 cells/mL, 3 mL/well) were cultivated in six-well plates for 24 h. After the treatment with test compounds (10 and 25 nm), C-A4 (10 and 25 nm), or DMSO (vehicle) for an additional 24 h, the cells were trypsinized, centrifuged (300 × *g*, 5 min, 4 °C), and fixed in 70% EtOH for min. 24 h. For FACS measurement, cells were washed in PBS and stained with propidium iodide solution (50 µg/mL PI, 50 µg/mL RNase A in 0.1% sodium citrate) for 30 min at 37 °C. The DNA content of at least 10000 single cells was determined using a Beckmann Coulter Cytomics FC500 flow cytometer (λ_{ex} = 488 nm, λ_{em} = 570 nm). CXP software (Beckman Coulter) was used to analyze the cells in the cell cycle phases (sub-G1, G1, S, G2/M).

Tube-formation assay

EA.hy926 endothelial hybrid cells were maintained for 24 h in EndoPrime low-serum endothelial medium and seeded (3 × 10^5 cells/mL, 50 µL/well) on basement membrane-like matrix Matrigel® on Ibidi µ-Slides. After treatment with test compounds for 4 h until tubular structures had formed in the control wells, results were documented using a Zeiss Axiovert 135 light microscope. Cell vitality was measured using the MTT assay and was above 75% compared to solvent-treated cells. Experiments were performed in triplicate.

Zebrafish angiogenesis assay

Transgenic zebrafish (*Tg(fli1:EGFP, casper* mutant) were bred at 28 °C.[16] After fertilization, the eggs were transferred to E3-medium (5 mm NaCl, 0.17 mm KCl, 0.33 mm CaCl, 0.33 mm $MgSO_4$, 0.01% methylene blue, pH 7.2), followed by incubation for 24 h. The chorion was manually removed, and the larvae were treated with test compounds or solvent in six-well plates (30 fish per concentration, 5 mL/well) for 48 h. The fluorescent vasculature was documented with a Leica MZ10F and Zeiss AxioCam MRrc. To quantify the angiogenesis, the sub-intestinal vein (SIV) area was measured using ImageJ and expressed as means ± SD with the control set to 100%. The significance of SIV reduction through substance treatment was assessed using one-way analysis of variance (ANOVA), *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, with Dunnett´s multiple comparison test (GraphPad Prism 9).

Luciferase-dependent MYB activity assay

Compounds were tested using the HEK-MYB-Luc reporter cell line, which allows a doxycycline-dependent induction of MYB expression and harbors an MYB-dependent luciferase reporter plasmid, as previously described[17,18]. After 16 h of substance treatment (0.0001-3 µM), luciferase activities were analyzed as described[11].

RESULTS

The test compounds 2A-R were obtained from 3-methoxyphenol, malononitrile, the corresponding aryl aldehyde, and a cat. amount of Et_3N in acetonitrile [Scheme 1]. Synthesis and analysis of the known compounds 2B, 2O, and 2R were as described[5,14]. The new compounds 2A, 2C-N, 2P, and 2Q were colorless solids. The structures of the test compounds were confirmed by NMR, IR, and MS analyses. The compounds are racemic mixtures, and no efforts to separate enantiomers were performed. The yields were generally low but acceptable considering the simple one-pot reaction and workup and the commercially available starting compounds [Table 1].

The new propargyl ether derivative 3 was prepared to conduct localization studies of this compound within cancer cells [Scheme 2]. 3-Propargyloxyphenol was obtained from the reaction of resorcinol with 1.2 equiv. propargyl bromide (80% in toluene) in DMF under basic conditions (K_2CO_3)[19]. Harsher conditions (reflux for 5 h in EtOH) were necessary to synthesize 3 by the described three-component reaction compared with the synthesis conditions of compounds 2A-R. 3 was obtained as an off-white solid in low yields only.

The antiproliferative activity of compounds 2A-R and 3 was evaluated in nine tumor and hybrid cell lines from six entities and compared with previously published data of the known compounds 1A and 1B [Table 2][13,20].

The presentation of the cytotoxicity screening results was simplified by determining the average IC_{50} values of all cell lines and sorting them based on their antiproliferative activity [Table 3]. The halogen atom did not have any major effect on the activity among the compounds with a 3-halo-4, 5-dimethoxyphenyl group (2A, 2B, and 2C). By contrast, in the group of 3, 5-dichloro- to 3, 5-diiodo-4-methoxyphenyl derivatives 2D, 2E, and 2F, a distinct increase in activity was observed from the dichloro compound 2D via the dibromo 2E to the diiodo derivative 2F. Several striking effects were observed in addition to this trend. Comparing the structure of 1C (coumarin-based) and 2F (7-methoxy-4*H*-chromene), a clear increase in antiproliferative activity based on the methoxychromene structure was observed, while the IC_{50} values of the 4*H*-naphtho(1, 2-*b*)pyran-3-carbonitriles (1A) and the 7-methoxy-4*H*-chromenes (2R) were both in the nanomolar range. Some compounds showed certain tumor cell line-specific activities, surpassing positive controls 1A and 1B. 2C was particularly active against HCT116 p53-deficient colon carcinoma cells, verapamil-treated KB-$V1^{Vbl}$ cervix carcinoma cells and MCF-7 breast cancer cells, whereas 2F and 2O were highly active against multidrug-resistant HT-29 colon carcinoma cells. The 1A-analog 2R showed slightly less overall activity but had a much stronger effect on MCF-7 breast cancer cells than 1A. The vinblastine-resistant KB-$V1^{Vbl}$ cells were treated with the P-glycoprotein (P-gp) blocker verapamil to identify synergy effects[21]. Compounds 2I, 2B, 2C, and 2E showed 12-, 14-, 20- and 320-fold lower IC_{50} values combined with verapamil (1 μM), assuming an inhibition of the efflux pumps increases the substance efficiency by blocked drug removal via the P-gp membrane transporter. A comparison of compounds 2B and 3, prepared for intracellular localization purposes, showed a substantial conformity of activity, indicating a similar mode of action and accumulation behavior in most tumor cell lines. However, compound 2B was more active against verapamil-treated KB-$V1^{Vbl}$ cells than against untreated cells and HeLa cells (which is the parent cell line of KB-3-1, from which KB-$V1^{Vbl}$ cells are derived), while 3 was more active against HeLa cells and KB-$V1^{Vbl}$ cells in the absence of verapamil than against verapamil-treated KB-$V1^{Vbl}$ cells.

The most active derivatives 2A-C and 2E-F and the positive control 1A were tested for their toxic effects on non-malignant HDFa cells [Table 3]. The selectivity index (SI) was calculated as a measure of selectivity toward cancer cells compared to non-malignant cells. 2B, 2C, and 2F displayed exceptionally high SI values, highlighting their potential as anticancer drug candidates.

Table 1. Yields of the syntheses of compounds 2A-R

Compound	Yield	Compound	Yield	Compound	Yield
2A	22%	2G	25%	2M	32%
2B	30%	2H	40%	2N	30%
2C	32%	2I	31%	2O	20%
2D	31%	2J	30%	2P	32%
2E	31%	2K	30%	2Q	32%
2F	30%	2L	32%	2R	30%

Table 2. IC_{50} values (in nM) of 2A-R and 3 in tumor cell lines.[a] 1A (LY290181) and 1B (Bcr-TMP) were used as positive controls

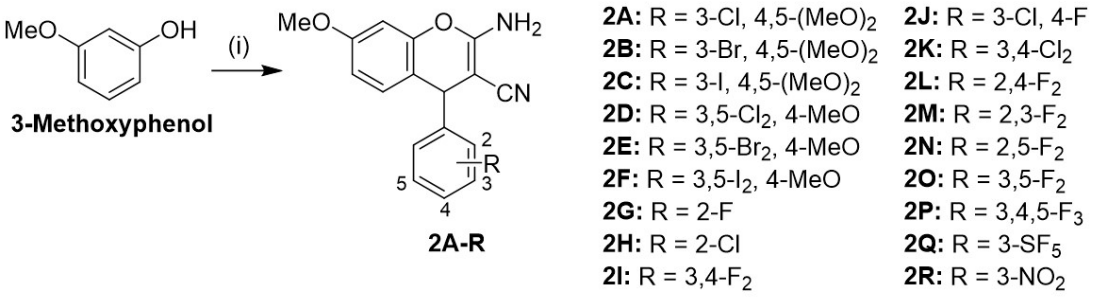
	EA.hy926	518A2	HCT-116	HCT-116 p53-/-	U87	HT-29	KB-V1^{Vbl}	KB-V1^{Vbl}[b]	HeLa	MCF-7	HDFa
1A[c]	30 ± 1	10 ± 1	8 ± 0.7	30 ± 3	70 ± 9	30 ± 3	20 ± 2	80 ± 2	40 ± 2	200 ± 20	40,700 ± 1500
1B[c]	20 ± 1	30 ± 0.1	20 ± 1.9	30 ± 2	5 ± 0.2	300 ± 40	30 ± 1	9 ± 1	10 ± 1	34 ± 2.5	-
1C[d]	2800 ± 70	1500 ± 100	3100 ± 100	2900 ± 60	5500 ± 300	2500 ± 200	3700 ± 60	3500 ± 80	-	3400 ± 200	-
2A	0.4 ± 0.06	4 ± 0.3	70 ± 5	70 ± 5	20 ± 3	300 ± 20	6 ± 0.5	0.9 ± 0.08	10 ± 0.4	40 ± 7	11,500 ± 1200
2B	0.8 ± 0.09	6 ± 0.2	100 ± 10	30 ± 3	80 ± 5	200 ± 20	10 ± 1	0.7 ± 0.06	2 ± 0.2	100 ± 8	> 50,000
2C	60 ± 5	20 ± 2	70 ± 1	6 ± 0.7	25 ± 2	200 ± 20	60 ± 5	3 ± 0.3	10 ± 0.9	20 ± 1	> 50,000
2D	1100 ± 70	2100 ± 200	2500 ± 200	1900 ± 200	3900 ± 400	1400 ± 100	2100 ± 100	2400 ± 100	700 ± 50	2400 ± 300	-
2E	20 ± 1	20 ± 2	20 ± 2	40 ± 3	20 ± 2	300 ± 2	3200 ± 300	10 ± 0.5	30 ± 3	80 ± 3	22,200 ± 2100
2F	20 ± 2	20 ± 0.4	30 ± 5	30 ± 1	50 ± 10	60 ± 1	70 ± 7	40 ± 2	30 ± 3	80 ± 4	> 50,000
2G	900 ± 90	700 ± 40	200 ± 20	300 ± 40	3200 ± 100	200 ± 10	700 ± 20	500 ± 20	400 ± 60	500 ± 40	-
2H	3700 ± 300	2000 ± 100	3700 ± 400	2800 ± 300	7400 ± 200	5300 ± 970	1900 ± 200	1600 ± 200	1400 ± 100	5100 ± 370	-
2I	700 ± 10	700 ± 5	800 ± 6	600 ± 6	3600 ± 500	800 ± 30	700 ± 30	60 ± 5	600 ± 30	700 ± 100	-
2J	400 ± 20	400 ± 10	300 ± 30	400 ± 40	1200 ± 70	500 ± 50	300 ± 20	210 ± 10	100 ± 6	150 ± 20	-
2K	600 ± 80	600 ± 7	300 ± 10	800 ± 20	8500 ± 300	1900 ± 100	1300 ± 100	600 ± 50	800 ± 70	1000 ± 50	-
2L	200 ± 10	500 ± 30	2500 ± 40	1600 ± 160	5100 ± 500	500 ± 50	3800 ± 400	1000 ± 100	1500 ± 70	> 50,000	-
2M	400 ± 10	300 ± 10	500 ± 50	200 ± 8	400 ± 60	200 ± 20	900 ± 90	100 ± 4	90 ± 2	200 ± 30	-
2N	4700 ± 900	90 ± 60	500 ± 50	200 ± 10	500 ± 100	400 ± 30	700 ± 70	200 ± 20	200 ± 20	300 ± 7	-
2O	40 ± 1	60 ± 6	1400 ± 50	40 ± 3	100 ± 10	70 ± 8	20 ± 2	60 ± 4	40 ± 4	400 ± 60	-
2P	400 ± 40	300 ± 30	300 ± 20	300 ± 20	1100 ± 90	200 ± 5	100 ± 5	100 ± 10	100 ± 20	400 ± 50	-
2Q	600 ± 20	300 ± 10	700 ± 40	2900 ± 10	900 ± 60	400 ± 30	1900 ± 100	2000 ± 100	400 ± 4	800 ± 30	-
2R	300 ± 9	30 ± 2	300 ± 30	200 ± 20	900 ± 50	200 ± 20	30 ± 0.4	70 ± 6	300 ± 20	10 ± 0.8	-
3	80 ± 2	60 ± 3	300 ± 10	90 ± 7	90 ± 3	2100 ± 300	80 ± 3	100 ± 3	40 ± 2	70 ± 4	-

[a]Means of min. four independent experiments (± SD); [b]plus verapamil; [c]Köhler *et al.*[20]; [d]Köhler *et al.*[13].

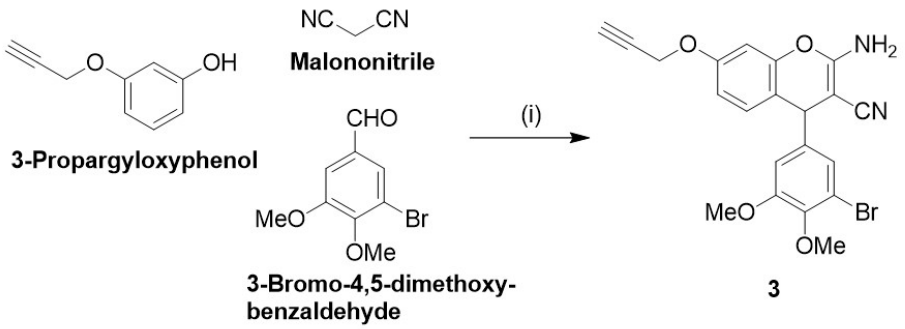
For a more detailed investigation of possible drug mechanisms, compounds 2A-C and 2F were tested for their influence on the cell cycle of 518A2 melanoma cells. FACS analysis at doses of 25 nM revealed a significant G2/M cell-cycle arrest for these compounds and the positive control combretastatin A4 (CA4), but to varying extents [Figure 2].

Table 3. Average IC_{50} value [nM] of the tested cancer and hybrid cell lines for compounds 1A-B, 2A-R, and 3, and SI (IC_{50} HDFa cells/ IC_{50} tumor cell average)

	2F	2C	2B	1A	2A	1B	2O	2R	3	2M	2P	2E	2J	2G, 2N, 2J, 2Q, 2K, 2D, 1C, 2H, 2L
Ø IC_{50} [nm]	43	47	49	52	53	53	203	227	321	330	330	374	396	> 500
SI	1163	1055	952	786	219	-	-	-	-	-	-	59	-	-



Scheme 1. Reagents and conditions: (i) Malononitrile; aryl aldehyde; cat. Et_3N; MeCN; rt; 3-16 h.



Scheme 2. Reagents and conditions: (i) cat. piperidine; EtOH; reflux; 5 h; 11%.

Among the compounds 2A-C, the 3-chloro-4, 5-dimethoxyphenyl derivative 2A showed more significant cell-cycle arrest than its 3-bromo and 3-iodo congeners 2B and 2C. However, an opposite effect was observed for the 3, 5-dihalo-4-methoxyphenyl derivatives 2D-F, where the 3, 5-diiodo-4-methoxyphenyl derivative 2F triggered by far the most significant arrest. The analogous 3, 5-dichloro (2D) and 3, 5-dibromo (2E) compounds induced only a slight increase in G2/M phase cells (data not shown). Substance 2F arrested about 74% of cells before or during mitosis and exceeded the C-A4 control. C-A4, 1A, and 1B caused G2/M arrest by microtubule depolymerization, thus preventing the formation of a functional spindle apparatus required for cell division[7,11,22].

Because of the structural similarity of the new cell cycle arresting compounds 2A-C and 2F to the known tubulin-depolymerizing agent C-A4, the effects on the microtubule cytoskeleton were investigated. Time-dependent imaging of 518A2 melanoma cells was applied to provide insights into the dynamics of the depolymerization process [Figure 3].

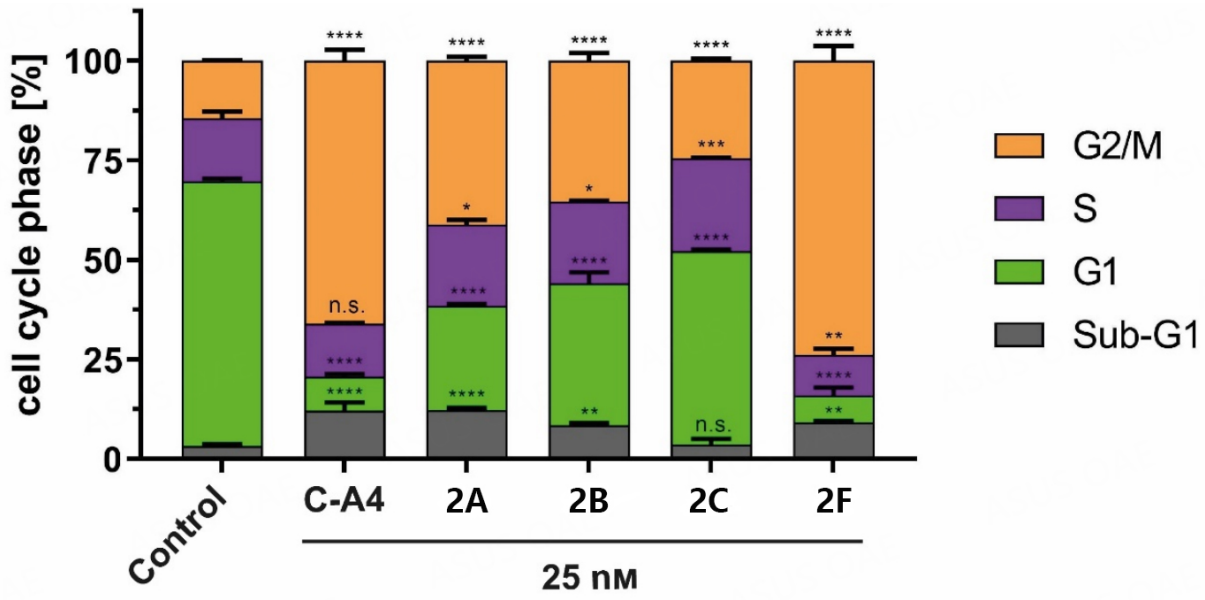


Figure 2. Cell cycle events of 518A2 melanoma cells treated with 2A-C and 2F (25 nM) for 24 h. Positive control (C-A4) and solvent (DMSO) were treated similarly to the substances. Measurements were carried out in triplicate and expressed as means ± SD with GraphPad Prism. Significance is expressed as n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ against control (two-way ANOVA, with Dunnett's multiple comparison test).

In solvent-treated cells, the tubulin cytoskeleton consists of fine filaments extending throughout the cytoplasm. Upon treatment with test compounds 2A-C and 2F, these filaments were initially shortened and fragmented, leading to their destruction and distribution within the cytoplasm. However, the timing of this disintegration process was highly dependent on the substance used. For instance, 2A-C showed a breakdown of the cytoskeleton after 6 h, whereas 2F led to a change in the microtubule structure after 1 h. A similar effect was observed for the C-A4 control after 30 min, which can be attributed to a faster uptake or higher target affinity.

The alkynyl derivative 3 was designed to investigate compound uptake and localization within tumor cells. The propargyl group allows orthogonal fluorescence labeling with 3-azido-7-hydroxycoumarin using a copper-catalyzed click reaction. After 10 min, a distinct increase in fluorescence was observed within the treated cells, with most of the fluorescence found in the cytoplasm [Figure 4]. This finding suggests an accumulation of 3 in the cytoplasm, supporting the hypothesis that cytoplasmic tubulin is the primary target for 3 and its close analogs used in this study. The conformity of the basic structure and the antiproliferative activity of 3 with compound 2 suggests a similar mechanism.

In addition to antiproliferative and cytotoxic effects, microtubule-destabilizing agents possess additional antitumor properties[23]. In the case of C-A4, anti-angiogenic and vascular disruptive effects have been demonstrated. In this context, the tube-formation assay is a suitable method to observe the effects on the two-dimensional (2D) vessel structures by EA.hy926 cells[24]. The inhibition of cell migration and the development of cell-cell junctions (necessary for tube formation) by 2A-C and 2F were investigated [Figure 5]. At 100 nM, all four test compounds showed anti-angiogenic effects on EA.hy926 cells seeded on Matrigel®. The cells could not form a cross-linked 2D structure within 4 h, as with the negative control or at 25 nM substance concentration. Even if cell-cell junctions were formed in isolated cases, most cells agglomerated due to their spatial proximity. In addition, rounding was observed in many cells, as in C-A4 treated cells, presumably because of microtubule-destabilizing effects.

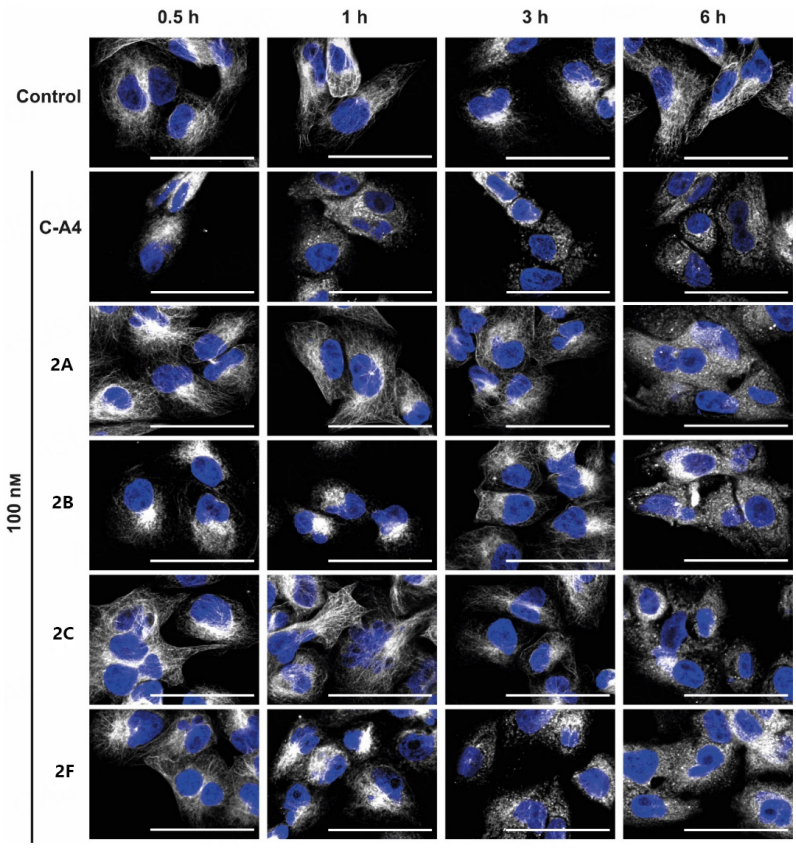


Figure 3. Immunofluorescence images of 518A2 melanoma cells treated with compounds 2A-C; 2F; and CA4 (100 nM) or vehicle (DMSO) for 0.5, 1, 3, and 6 h. Representative images (of two experiments) illustrate stained microtubules (white) and nuclei (blue). The scale bar corresponds to 100 µm, magnification of 630×.

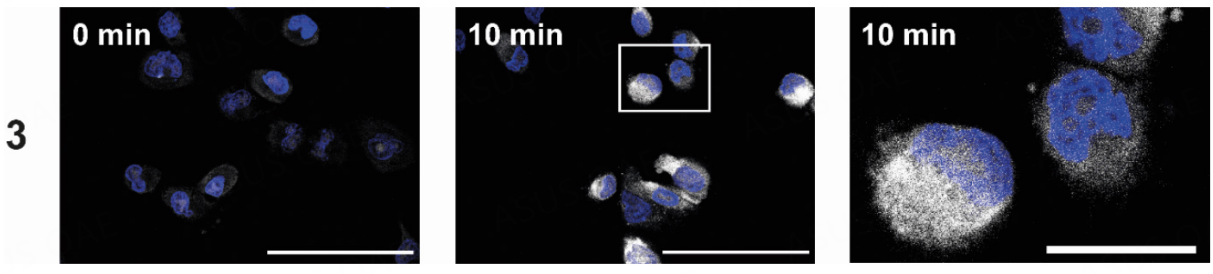


Figure 4. Intracellular localization of 3 (25 µM) using melanoma cells (518A2) after 10 min. The uptake was visualized using a Cu(I)-catalyzed reaction with 3-azido-7-hydroxycoumarin (white), and the nuclei were counterstained (Nuclear Green, blue). The right image shows the magnified section marked with a white box. The experiment was carried out in duplicate. The scale bar corresponds to 100 µm (left) or 25 µm (right), magnification of 630×.

The development of blood vessels is based on a complex mechanism with various regulators, which are essential targets for the treatment of tumor growth. Angiogenesis can be seen in the embryonal development of zebrafish larvae, where the SIV can be used to measure anti-angiogenic effects[25]. After exposure of 24-h-old zebrafish embryos to substances 2A-C and 2F or positive control axitinib for 48 h, we

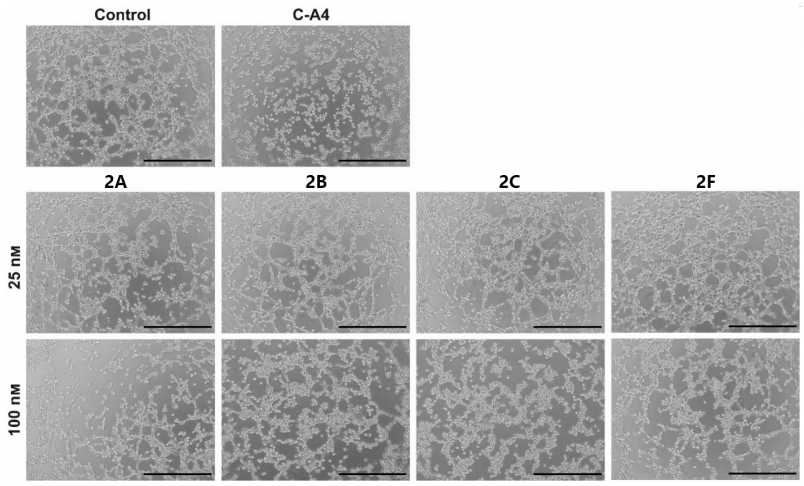


Figure 5. Images show EA.hy926 cells seeded on Matrigel® after 4 h treatment with substances 2A-C; 2F (25, 100 nM); and C-A4 (25 nM) or vehicle (DMSO). Representative images of a min. of two experiments. The scale bar corresponds to 500 µm, magnification of 100×.

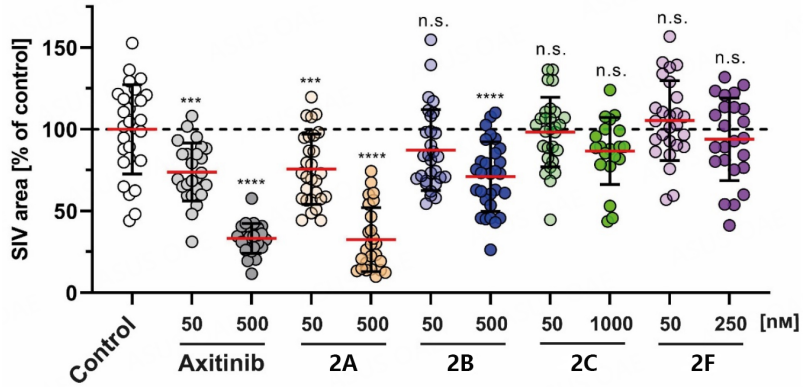


Figure 6. Effects of 2A; 2B (50, 500 nM); 2C (50, 1000 nM); and 2F (50, 250 nM) on the SIV growth of zebrafish larvae (24 hpf) after treatment (48 h). Positive controls used axitinib (50, 500 nM). Negative controls used equivalent amounts of DMSO. The SIV area was quantified using ImageJ and expressed as mean ± SD of at least 20 zebrafish. The significance is expressed as n.s. $P > 0.05$; ***$P < 0.001$; ****$P < 0.0001$ against control (one-way ANOVA, with Dunnett´s multiple comparison test).

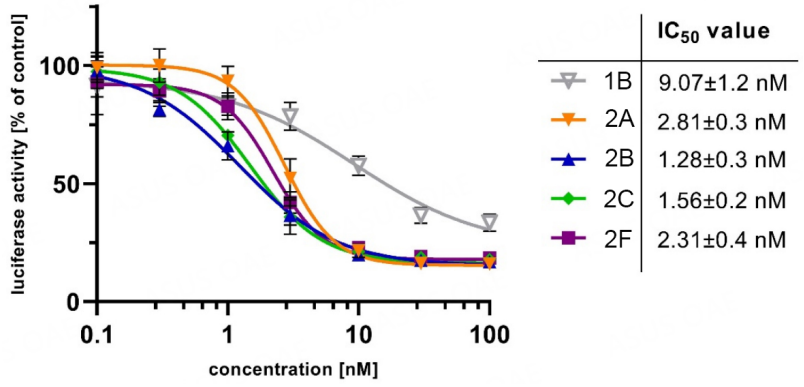


Figure 7. Inhibition of MYB activity in HEK-293 cells containing the reporter plasmid pGL4-5xMRE(GG)-Myc and the expression vector for MYB-2KR, upon treatment with compounds 1B; 2A-C; and 2F (0.1-100 nM) for 16 h. IC_{50} values were calculated with at least four independent experiments using GraphPad Prism 9.

measured the SIV area and compared it to solvent-treated fish [Figure 6]. Here, 2F showed no significant change in blood vessel growth, and 2F had a toxic effect above 250 nM. 2B and 2C showed no SIV decrease in the tolerated concentration range. However, 2A inhibited angiogenesis to an extent comparable to the known inhibitor axitinib[26]. This finding suggests a different mechanism of action for 2A, a bimodal compound with selective antiproliferative properties against cancer cells and angiogenesis-inhibiting features.

Due to their structural similarity to the potent MYB inhibitor 1B (data to be published elsewhere), compounds 2A-C and 2F were tested for their MYB-inhibitory activity [Figure 7]. Inhibition of MYB activity by 2A-C and 2F (1.28-2.81 nM) was superior to the inhibition by reference compound 1B (9.07 nM). Because the values are very close, it is challenging to identify a structure-dependent activity trend; however, the activity was in the order 2B > 2C > 2F > 2A.

DISCUSSION

This study's objective was to develop and optimize the lead structures 1A, 1B, and 1C. For this reason, we reduced the size of the benzo[*h*]chromene backbone to a 7-methoxy-4*H*-chromene structure, which led to increased activity. In addition, various phenyl substituents were tested to optimize the lead structure. Initial cytotoxicity studies against nine cancer cell lines confirmed that the C-A4-derived 3-iodo-, 3-bromo-, and 3-chloro-4, 5-dimethoxyphenyl motifs (2A-C) and the analogous 3, 5-diiodo- and 3, 5-dibromo-4-methoxyphenyl derivatives (2E-F) are excellent pharmacophores with selectivity against malignant cells. Only the 3, 5-dichloro-4-methoxyphenyl stands out with only micromolar IC_{50} values. Interestingly, differences in the specificity for HeLa cells, derived KB-V1^{Vbl} cells, and KB-V1^{Vbl} cells treated with the P-gp inhibitor verapamil were observed for the close analogs 2B and 3. 2B is more active against the parent HeLa cells than against the vinblastine-resistant KB-V1^{Vbl} cells. In contrast, the addition of verapamil strongly sensitized the KB-V1^{Vbl} cells to treatment with 2B, leading to a higher activity of 2B against verapamil-treated KB-V1^{Vbl} cells than against HeLa cells. A comparable hypersensitizing effect of verapamil was observed for KB-V1^{Vbl} cells treated with 2A, 2C, and 2I, surpassing their activity against HeLa cells, which may have explanations beyond mere P-gp inhibition by verapamil. The hypersensitivity effects of resistant P-gp-overexpressing cancer cells upon verapamil treatment were reported (e.g., based on disrupted energy homeostasis upon ATP depletion), which might lead to enhanced anticancer activity combined with other active drug candidates such as 2A-C (but not 2F) compared with their activity against related cell lines without (overexpressed) P-gp transporter[27]. For unknown reasons, compound 3 showed only slightly reduced activity against KB-V1^{Vbl} cells when combined with verapamil than against HeLa cells.

In addition, a reversal of P-gp- and BCRP-mediated resistance was documented for the 3-chloro-4-fluoroanilino-derivative gefitinib, an approved EGFR inhibitor[28,29]. However, the new 3-chloro-4-fluorophenyl derivative 2J appears to be a substrate of P-gp. Among the remaining fluorophenyl derivatives, 2, 3-difluorophenyl 2M and 2, 5-difluorophenyl 2N were identified as P-gp substrates, while 3, 5-difluorophenyl 2O and 3, 4, 5-trifluorophenyl 2P showed no dependence on P-gp, indicating a considerable influence of the fluoro-substitution pattern on the activity against P-gp-overexpressing cells (also compared with the effects of 3, 4-difluorophenyl derivative 2I mentioned above).

Because P-gp tends to have relatively hydrophobic substrates with aromatic rings, substituting methoxy groups with halogen atoms may already have an efflux-attenuating effect due to increased hydrophilicity[30]. Treatment with P-gp modulators can provide more significant cytotoxicity by reducing effective concentrations by 100-fold (paclitaxel) or 351-fold (vinblastine) in MDR colorectal cancer SW620/Ad300 cells[31,32].

The test compounds were obtained and tested as racemic mixtures. An increase in activity might be achieved by the separation of the enantiomers of the active derivatives and evaluating the separated enantiomers via MTT assay to identify any enantiomers which are more active or less active than the mixture. Instead of separating racemic mixtures, chiral synthetic procedures (e.g., using chiral organic bases instead of piperidine or triethylamine) might be applied to generate enantiopure compounds for biological testing.

The evaluation of the four most active test compounds (2A-C and 2F) revealed a correlation between the rate of microtubule destruction and the cell-cycle arrest in the G2/M phase in 518A2 melanoma cells. Compared with C-A4, 2F showed almost equal efficacy and led to early morphological changes in the microtubules (i.e., within 1 h). Localization of the structurally related alkynyl derivative 3 in the cytosol confirmed the accumulation near the cytoplasmic target. As previously demonstrated for the controls 1C and C-A4, the influence on angiogenesis (essential for tumor growth and metastases) was investigated for the test substances[13,33]. Using the 2D tube-formation assay, the concentration-dependent impairment of cell motility and intercellular junctions could be demonstrated, probably due to the damaged tubulin cytoskeleton. Nevertheless, this model can only partially simulate the complex mechanisms of blood vessel formation, which is why the substances were also tested for anti-angiogenic effects in zebrafish. This assay offers the possibility of estimating toxicity in a vertebrate and investigating the direct influence on the angiogenesis of the so-called SIVs. Surprisingly, only 2A showed a significant decrease in SIV growth, which occurred independently of its antiproliferative and microtubule-associated effects. As a third potential target, the inhibitory effect on the transcription factor MYB was tested and revealed the enhancement by three- to seven-fold compared to the previously discovered inhibitor 1B[11]. Overexpression of MYB in leukemias and various solid cancers such as colon and ER-positive breast cancers contributes to their development and thus represents a valuable target[34-39]. The rapid development of resistance to selective chemotherapeutics (e.g., kinase inhibitors, including RAF inhibitors) is a problem that can be overcome by addressing various targets by drugs with dual or multimodal mechanisms of action such as dual BRAF/HDAC inhibitors[40,41]. In this context, compound 2A represents a promising scaffold that can be used for further optimization and advanced stages of preclinical anticancer testing.

DECLARATIONS

Authors' contributions

The manuscript was written through the contributions of all authors.
Conceptualization: Köhler LHF, Klempnauer KH, Schobert R, Biersack B
Methodology: Köhler LHF, Reich S, Yusenko M, Biersack B
Formal analysis: Köhler LHF, Yusenko M, Biersack B
Resources: Klempnauer KH, Begemann G, Schobert R
Data curation: Köhler LHF, Yusenko M, Biersack B
Writing - original draft preparation: Köhler LHF, Biersack B
Writing - review and editing: Klempnauer KH, Begemann G, Schobert R
Supervision: Klempnauer KH, Schobert R, Biersack B
Funding acquisition: Klempnauer KH
All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data supporting the findings of this study are available from the authors upon request.

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Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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Cancer Drug Resistance

Review

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Nano-TRAIL: a promising path to cancer therapy

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Abstract

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand, also called apo-2 ligand (TRAIL/Apo-2L), is a cytokine that triggers apoptosis by binding to TRAIL-R1 (DR4) and TRAIL-R2 (DR5) death receptors. Apoptosis occurs through either the extrinsic or intrinsic pathway. The administration of recombinant human TRAIL (rhTRAIL) or TRAIL-receptor (TRAIL-R) agonists promotes apoptosis preferentially in cancerous cells over normal cells *in vitro*; this phenomenon has also been observed in clinical studies. The limited efficacy of rhTRAIL in clinical trials could be attributed to drug resistance, short half-life, targeted delivery issues, and off-target toxicities. Nanoparticles are excellent drug and gene delivery systems characterized by improved permeability and retention, increased stability and biocompatibility, and precision targeting. In this review, we discuss resistance mechanisms to TRAIL and methods to overcome TRAIL resistance by using nanoparticle-based formulations developed for the delivery of TRAIL peptides, TRAIL-R agonists, and TRAIL genes to cancer cells. We also discuss combinatorial approaches of chemotherapeutic drugs with TRAIL. These studies demonstrate TRAIL's potential as an anticancer agent.

Keywords: TRAIL, cancer cells, nanoparticles, apoptosis, nanomedicine

INTRODUCTION

Cancer is the aberrant growth of cells capable of invading and metastasizing to other body parts. It is one of the leading causes of death, with approximately 10 million deaths expected globally by 2020, according to

the World Health Organization. There are various treatment options, including surgery, chemotherapy, targeted therapy, radiation therapy, immunotherapy, and hormone therapy[1]. One branch of chemo/radiotherapy is targeted therapy that targets the tumor alone (for example, tumor vasculature or intracellular organelles, leaving the surrounding cells unaffected). This approach results in precise treatment of the tumor alone, reducing the drawbacks associated with the process[2]. Among the many targeted therapies is Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand, also known as Apo-2 ligand (TRAIL/Apo-2L).

TRAIL/Apo-2L is a type II transmembrane protein of the TNF superfamily of ligands. TRAIL binds to TRAIL-Receptor 1/Death Receptor 4 (TRAIL-R1/DR4), TRAIL-Receptor 2/Death Receptor 5 (TRAIL-R2/DR5), TRAIL-Receptor 3/Decoy Receptor 1 (TRAIL-R3/DcR1), and TRAIL-Receptor 4/Decoy Receptor 2 (TRAIL-R4/DcR2). It also binds to osteoprotegerin (OPG), which is soluble[3,4]. TRAIL selectively promotes apoptosis in cancer cells by the extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) pathways[5-7]. Apoptosis is a well-ordered and well-coordinated cellular process that maintains homeostasis in physiological and diseased conditions. In the extrinsic pathway, trimerization and subsequent receptor activation occur upon binding of TRAIL to its respective DR4 and DR5 death receptors, which leads to Death Inducing Signaling Complex (DISC) formation[8]. The intrinsic pathway includes mitochondrial outer membrane permeabilization (MOMP) and apoptosome formation[9,10]. Intrinsic and extrinsic mechanisms activate the executioner caspases (caspase-3/7), which cause DNA degradation and result in cell death. Here, we elaborate on the TRAIL-induced apoptotic pathways, resistance mechanisms exhibited by cancer cells to TRAIL therapy, and current approaches to overcome the resistance and utilize TRAIL as a potential cancer therapeutic agent.

TRAIL and TRAIL receptors

TRAIL is a transmembrane protein belonging to the type II superfamily of ligands, coded by the gene TNFSF10 positioned at 3q26 on human chromosome number 3. In its monomeric form, TRAIL comprises 281 amino acids with a molecular mass of approximately 32.5 kDa. A loop formed by 12-16 amino acids near the N-terminus is critical for binding of TRAIL to its receptors and its cytotoxic activity[11]. TRAIL expression as a homotrimer is maintained by a zinc ion bound to cysteines necessary for its stability, solubility, and bio-activity[12]. TRAIL associates with DR4, DR5, DcR1, and DcR2, composed of a cysteine-rich domain (CRD), a transmembrane domain, and a death domain (DD), of which CRDs are extracellular, and DDs are intracellular [Figure 1]. The presence or absence of these domains characterizes TRAIL receptor structure and function. The type I transmembrane proteins (DR4 and DR5) are TRAIL agonists, whereas DcR1 and DcR2 are antagonists. DR4 and DR5 include an intracellular DD responsible for apoptosis induction. Unlike DR4 and DR5, DcR1 and DcR2 cannot trigger apoptosis due to the lack of a DD in the former and the presence of a non-functional truncated DD in the latter[13]. TRAIL also binds to osteoprotegerin (OPG), a receptor secreted as a soluble dimer. TRAIL binding to OPG occurs with low affinity. Other TNF family members bind with greater affinity to OPG, activating bone remodeling pathways[14].

TRAIL-induced apoptosis: extrinsic and intrinsic pathways

DR4 and DR5 are cell surface death receptors that include a cytoplasmic DD. In the extrinsic apoptosis pathway, TRAIL binds to its respective death receptors, resulting in the recruitment via homotypic interactions of the Fas-associated death domain (FADD), an adapter protein to their intracellular death domains. FADD then recruits initiator caspase-8 and caspase-10 to its death effector domain (DED) region, forming the multi-protein DISC. The activation of caspases-8 and -10 leads to cleavage and activation of effector caspase-3, -6, and -7, resulting in cell death [Figure 2][15-18].

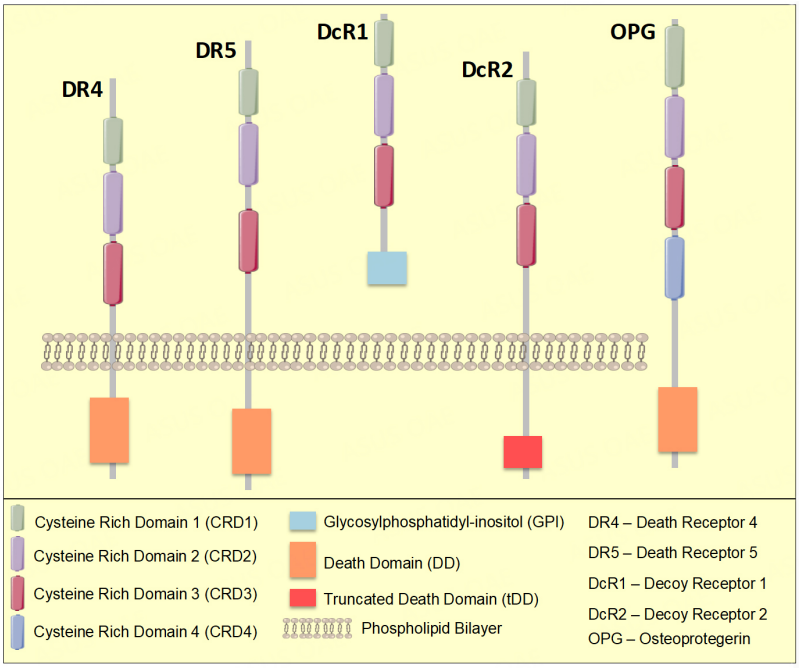

Figure 1. Structure of TRAIL receptors. TRAIL binds to death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) at the extracellular domains. The death domains (DD) on the cytoplasmic side lead to recruitment of other DD containing proteins that lead to apoptosis. TRAIL decoy receptors lack the cytoplasmic DD or have a truncated DD which fails to trigger apoptosis. OPG is a soluble receptor of TRAIL with low affinity.

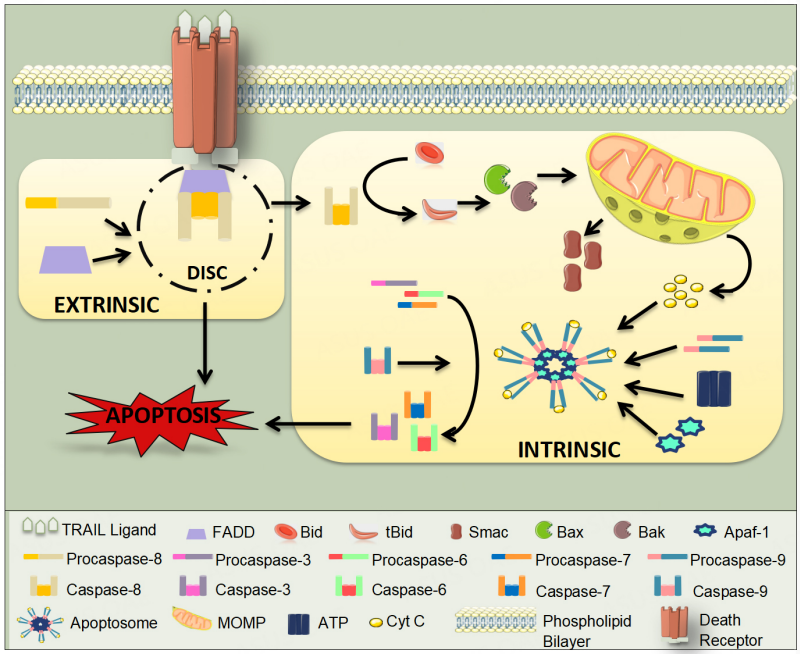

Figure 2. TRAIL-Induced Apoptosis: Cross talk between the Extrinsic and Intrinsic Pathways. TRAIL binding to death receptors leads to its trimerization on the cell surface and formation of DISC. Caspase-8 gets activated in the DISC and triggers the extrinsic pathway of apoptosis. Activated caspase-8 also leads to activation of the mitochondrial pathway or intrinsic pathway of apoptosis resulting in activation of caspase-9 and executioner caspases-3 and -7. Several checkpoints of apoptosis, like increase in anti-apoptotic proteins (IAPs), Bcl-xLetc are also represented at various stages of apoptosis. ATP: Adenosine triphosphate; DISC: Death Inducing Signaling Complex; FADD: Fas-associated death domain; MOMP: mitochondrial outer membrane permeabilization.

DNA damage, loss of survival factors, and cell cycle checkpoint defects trigger the intrinsic apoptosis pathway; the extrinsic and the intrinsic pathways are interrelated. In the intrinsic pathway, there is a cleavage of BH3-interacting domain death agonist (Bid) to truncated Bid (tBid)[19] in the presence of activated caspase-8 that brings about tBid translocation to the mitochondria and causes MOMP through activation of B-cell lymphoma 2 (Bcl-2) associated x-protein (Bax) and Bcl-2 homologous antagonist/killer (Bak). Mitochondria subsequently release cytochrome C[20] and mitochondria-derived activator of caspase (Smac)[21] into the cytosol, where cytochrome C interacts with adenosine triphosphate (ATP) and apoptotic peptidase-activating factor-1 (Apaf-1) to associate with initiator pro-caspase-9 into a signaling complex. This is an apoptosome where caspase-9 becomes activated and activates effector caspases-3, 6, and 7 to trigger apoptosis [Figure 2][8,10,22].

TRAIL AND CANCER THERAPY

TRAIL is a potential candidate for targeted therapy in cancer due to its selective induction of apoptosis via death receptor-4 and -5 expressed on the surface of target cells. The two primary treatment methods are the administration of recombinant TRAIL protein (small peptide or full-length protein) or TRAIL-receptor (TRAIL-R) agonists[23]. However, the short half-life of TRAIL protein in serum and the inefficacious administration of the agonists *in vivo* have hampered its therapeutic use[24]. Moreover, many cancerous cells have been discovered to be TRAIL-resistant[25]. Several groups have tried to decipher the resistance mechanisms in various cancers and various ways to sensitize cells to TRAIL[26-30]. Several explanations have been offered for TRAIL resistance, some of which are discussed below.

Mechanisms of cancer cell resistance to TRAIL

TRAIL receptor expression and status

TRAIL induces apoptosis by binding to DR4 and DR5 death receptors. Loss of DR4 and DR5 expression because of constitutive endocytosis results in TRAIL resistance and is observed in some breast cancer cells. The comparison of six cell lines of human breast cancer (SKBR3, MDA-MB-468, MCF7, MDA-MB-231, T47D, and BT474) treated with recombinant human TRAIL (rhTRAIL) and antibodies against death receptor 4 (DR4) and death receptor 5 (DR5) for the apoptotic response showed the loss of expression of DR4 and DR5 in some cell lines that accounted to their antibody resistance. The derangement of clathrin-dependent endocytosis signaling elements (adapter protein 2 and clathrin) by pharmacologic inhibitors like phenyl arsine oxide, PAO (a general inhibitor of endocytosis), and chlorpromazine (an inhibitor of clathrin-mediated endocytosis) restored the death receptors' expression on the cell surface, making TRAIL-resistant cells susceptible to TRAIL-induced apoptosis [Figure 3][31]. In another study, DR5-B, a receptor-selective TRAIL variant, caused independent internalization of DR5; the comparison of the kinetics of TRAIL-mediated internalization and subsequent DR4 and DR5 recycling in sensitive (HCT116 and Jurkat) and resistant (HT-29 and A549) tumor cell lines of various origins revealed that TRAIL stimulated DR4 and DR5 receptor internalization in a dose-related manner. This expression of the receptor on the cell surface was restored after TRAIL internalization and elimination observed by the addition of cycloheximide and brefeldin A, which hindered the process, suggesting that death receptors undergo constitutive endocytosis[32].

Apart from receptor endocytosis, mutations in death receptors[33] and expression of decoy receptors lacking the functional intracellular DD may play a key role in apoptosis induced by TRAIL[34]. DcRs, especially decoy receptor 4, might compete for binding to TRAIL or may disrupt trimerization of the death receptors when co-expressed on the same cell[35]. Post-translational modifications of the receptors also influence the sensitivity of the cells to TRAIL. *O*-glycosylation of TRAIL-R2 was found to control cell sensitivity to TRAIL[36], while *N*-glycosylation of TRAIL-R1 increased its apoptotic efficacy[37].

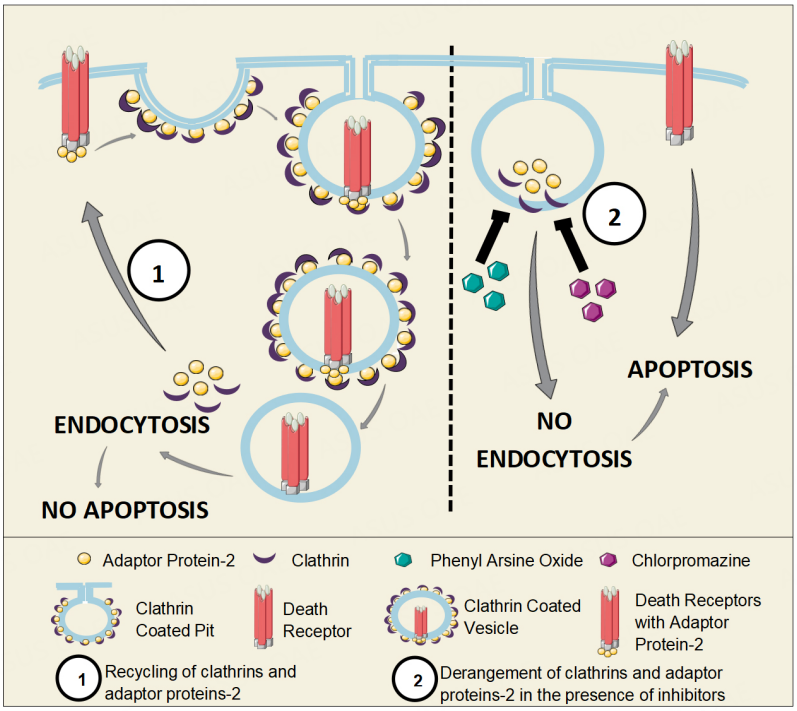

Figure 3. TRAIL Receptor endocytosis. Death receptors are constitutively recycled by clathrin mediated endocytosis. Lack of death receptors leads to TRAIL resistance. After endocytosis of the receptor, clathrin and adaptor proteins (AP2) are recycled to the cell membrane to continue with more endocytosis of the receptors. Pharmacological inhibitors like PAO or chlorpromazine prevent the arrangement of clathrin and AP2 at the vesicles, hence prevent endocytosis, resulting in persistent expression of the death receptors at the surface and increased apoptosis.

FADD defects and caspase-8

Cancer cells require FADD (an adapter protein) and caspase-8 for apoptosis mediated by DR4 and DR5. FADD recruitment by TRAIL results in the activation of pro-caspase-8, DISC formation, and apoptosis [Figure 2]. Defective FADD proteins do not allow TRAIL to recruit FADD and inhibit initiator caspases activation, resulting in TRAIL resistance. Experiments on FADD-deficient$^{(-/-)}$ mouse embryonic fibroblasts revealed that DR4 uses a FADD-independent apoptotic mechanism[38]. In another study, the transfection of mouse DR4/5, human DR4, or human DR5 into FADD-deficient mouse embryonic fibroblast cells conferred resistance to TRAIL-mediated cell death. However, they were vulnerable to TRAIL-mediated death when heterozygous FADD$^{(+/+)}$ or FADD$^{(-/-)}$ cells were reconstituted with a FADD retroviral construct[39].

Overexpression of cFLIP

The non-functional pro-caspase homolog and an anti-apoptotic protein known as cellular Flice-like inhibitory protein (cFLIP) is crucial in controlling TRAIL-induced apoptosis[40]; cFLIP binds to FADD and prevents recruitment of caspase-8/-10 to DISC, thereby inhibiting their activation [Figure 2][41-43]. In one study, DR5-positive and TRAIL-resistant IGR cells expressed enhanced TRAIL sensitivity with the downregulation of cFLIP. In TRAIL-sensitive and RPM-EP melanoma cells expressing DR5, TRAIL-mediated apoptosis inhibition was observed; due to the expression of cFLIP, TRAIL-R1 negative melanoma cells could not undergo apoptosis induced by TRAIL[44]. A study on epithelial-mesenchymal transition (EMT) showed that high expression of cFLIPs in the exogenous region caused resistance to apoptosis triggered by TRAIL, and deletion of cFLIPs was sufficient to overcome TRAIL resistance in carcinoma cell lines; when ML327 (an isoxazole-based small chemical) was induced into an immortalized mouse mammary epithelial cell line, there was a partial reversal of TGF-β-induced EMT[45].

CARP-dependent degradation of active caspase-8

CARPs are caspase-8 and -10-associated ring proteins that belong to a class of apoptotic inhibitors that bind to and negatively control caspases. Active caspases are essential in cancer cell death. The overexpression of CARPs in cancerous cells leads to the degradation of active caspases, which in turn causes a rise in the levels of cFLIP and, subsequently, TRAIL resistance. In one study, parental DLD1 human colon cancer cells developed TRAIL resistance after siRNA-mediated suppression of caspase-8 expression. When caspase-8 protein expression was restored through stable transfection, the DLD1/TRAIL-R cell line regained its TRAIL sensitivity, suggesting that the DLD1/TRAIL-R cells' TRAIL resistance may be due to low levels of caspase-8 protein expression. A 30%-50% increase in CARP-1 and -2 mRNA levels was observed in DLD1/TRAIL-R compared to a DLD1 cell line[46]. In another study, RNA interference-mediated silencing of CARPs resulted in H460 human lung cancer cell sensitization to death ligands due to activation of caspases; caspase-8 and -10 cleavage in reaction to the ligands were elevated in H460 cells after CARP-1 or -2 siRNA treatment and TNF-α/cycloheximide or TRAIL exposure[47].

Loss of Bax/Bak function (Bax Mutations)

The Bcl-2 antagonist/killer (Bak), and Bcl-2-associated X-protein (Bax), members of the Bcl-2 family, are core regulators of the intrinsic apoptotic pathway. Bax and Bak oligomerize and form pores in the mitochondrial outer membrane through which cytochrome C is released [Figure 2]. Cytochrome C triggers the intrinsic pathway[48-50]. A study of TRAIL-resistant leukemia cells lacking Bax and Bak showed no release of apoptogenic proteins from mitochondria. When Bax was transduced into Bax/Bak deficient leukemic cells, they exhibited sensitivity to TRAIL[51]. Cells lacking functional Bax treated with the chemopreventive agent sulindac and other non-steroidal anti-inflammatory agents completely deactivated the apoptotic pathway in human colorectal cancer cells. Loss of Bax did not affect TRAIL-induced caspase-8 activation or Bid cleavage but inhibited the mitochondrial release of Smac/Diablo and cytochrome C[52].

Bcl-2/Bcl-xL overexpression

B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL) are the anti-apoptotic Bcl-2 family proteins that inhibit mitochondria-dependent cell death pathways by hindering the movement of Bax from the cytosol to mitochondria [Figure 2]. In response to TRAIL treatment, apoptosis was reduced by Bcl-2 overexpression, causing TRAIL resistance[53-55]. Combining TRAIL with the protein synthesis inhibitor cycloheximide improved the sensitivity of the vector control cells to the triggering of apoptosis in SH-EP neuroblastoma cells[56]. Overexpression of Bcl-2 and Bcl-xL resulted in the reduction of TRAIL-induced cleavage of caspase-8 and Bid, cleaved blockage of caspase-3, -7, and -9 in non-small-cell lung cancer (NSCLC) and the cleavage of caspase substrates like PARP in glioblastoma, neuroblastoma, and breast cancer cell lines[57]. Bcl-2 overexpression inhibited TRAIL-induced Bax activation, loss of MOMP, and cleavage of caspase-8 into p43/p41 and p18 active fragments, cleavage of Bid, and processing of the caspase-3 p20 fragment into the p17/p12 active fragments in SH-EP neuroblastoma cells[58]. Bcl-xL overexpression was found in T47D and SKBR3 breast cancer cell lines resistant to TRAIL. Silencing Bcl-xL increased TRAIL-induced activation of caspase-3/7 in the estrogen receptor-positive cell line T47D and the HER2-amplified cell line SKBR3[59].

Overexpression of inhibitor of apoptosis proteins

Caspase activation is crucial for apoptosis. The family of anti-apoptotic proteins includes baculovirus repeat domain-containing proteins, also called inhibitor of apoptosis proteins (IAPs). These proteins consist of a (BIR) domain and a zinc ion binding site that coordinates protein-protein interaction and regulates the

activity of caspases. IAPs cause TRAIL resistance by inhibiting the activation of caspases downstream [Figure 2], thus blocking cell death[60-62]. In one study, when rhTRAIL alone or combined with Smac mimetics GDC-0152 or birinapant was used to treat non-iodine-retaining follicular thyroid carcinoma (FTC) cell lines, FTC133 and TT2609-bib2 resulted in FTC cell lines sensitization to apoptosis induced by TRAIL through cIAP1/2 degradation[60]. In another study, TRAIL sensitivity was restored in resistant cells and primary leukemic blasts upon treatment with XIAP inhibitors. The impacts of numerous anti-leukemic drugs could be used to overcome TRAIL resistance in leukemic cells via XIAP downregulation[63].

Reduction in smac/diablo release

A mitochondrial protein called the second mitochondria-derived activator of caspases (Smac) or direct inhibitor of apoptosis-binding protein with low pI (Diablo) binds to XIAP and eliminates the inhibitory effect of XIAP on caspase activation. Reduced release of Smac/Diablo causes a rise in the concentration of IAPs, inhibiting apoptosis[64-66]. SH122, a Smac-mimetic, significantly sensitized prostate cancer cell lines (DU145 and LNCaP) to TRAIL[67]. TRAIL-resistant lines associated with higher levels of XIAP released more Smac/Diablo than TRAIL-sensitive cells[68].

Activation of various mitogen-activated protein kinases/nuclear factor-kappa B subunits

Mitogen-activated protein kinases (MAPK) and nuclear factor-kappa B (NFkB) show opposing activities concerning TRAIL-induced cell death. The association of truncated Bid (tBid) with mitochondria to release cytochrome C is inhibited by the MAPK pathway. NFkB activation results in elevated levels of the anti-apoptotic proteins Bcl-xL and XIAP, leading to TRAIL resistance. NFkB suppression decreased resistance to TRAIL in various cancers[69,70]. Lovastatin, an NFkB inactivator, sensitized human glioblastoma cell lines (A172 and U87) that were TRAIL-resistant. The sensitization to TRAIL is attributed to increased levels of DR5 and dysregulation of the MAPK pathway[71]; c-Rel, an NFkB subunit regulates TRAIL-induced apoptosis in pancreatic ductal adenocarcinoma cells and transfection with siRNA against c-Relinduced apoptosis in TRAIL-resistant cells[72].

Akt pathway activation

The phosphatidylinositol 3-kinase-protein kinase B (PI3K-Akt) pathway is an intracellular signal transduction mechanism that stimulates cell growth and angiogenesis upon extracellular signals. Akt overexpression causes cancer cells to become highly resistant to TRAIL, while Akt knockdown causes resistant cancer cells to become more susceptible[73]. Inhibiting the Akt pathway with LY294002 (a PI3K inhibitor) or Akt knockdown sensitized TRAIL-resistant T47D breast cancer cells through cleavage of PARP[74]. Treatment of TRAIL-resistant MB-IT R and NALM-24 R (acute lymphoblastic leukemia cells) with LY294002 and TRAIL resulted in the activation of pro-caspase-8 and caspase-3[75]. Phosphatase and tensin homolog (PTEN), a tumor suppressor, negatively regulated the Akt pathway; PTEN-deficient cells were more resistant to TRAIL than PTEN(+/+) mouse prostate epithelial cells. Overexpressing a mutant PTEN made PTEN-positive cells resistant to TRAIL, suggesting that PTEN plays a part in TRAIL sensitivity. Overexpression of mutant PTEN showed increased resistance to TRAIL in T47D breast cancer cell lines resistant to TRAIL[74]. In SH-EP neuroblastoma cells, silencing the PI3K subunits p110 α and p110 β caused a selective downregulation in Akt phosphorylation. Combining TRAIL and PI103 (a PI3K inhibitor) led to an increase in broken caspases-8, -3, and -9, the conversion of Bid into tBid, and a decrease in the protein levels of Mcl-1, XIAP, survivin, cFLIPL, and cFLIPS[58]. El-Diery and colleagues described the role of PI3-Akt signaling in TRAIL- and radiation-induced gastrointestinal apoptosis. Activation PI3/Akt pathway protected gut cells from TRAIL-induced apoptosis but not from IR-induced apoptosis[76]. Akt activation reduced the sensitivity of epithelial ovarian cancer cells to TRAIL. PI3K or Akt inhibitors sensitized TRAIL-resistant SKOV3ip1 and COV2 cells. In TRAIL-resistant cells, low levels of Bid were observed due to overexpression of Akt[77].

Studies of TRAIL in cancer clinical trials

Because TRAIL triggers apoptosis specifically in cancer cells as opposed to tumor cells, several formulations of TRAIL protein and agonistic antibodies were tried in animal models and in clinical trials. Recombinant human TRAIL preferably promoted programmed cell death in cancerous cells over normal cells and exhibited minimal to no toxicity when injected systemically in animals[78]. This finding resulted in several treatment trials of biological agents targeting TRAIL receptors, including agonistic antibodies to DR4, DR5, and rhTRAIL[79] [Table 1]. The clinical evaluation of TRAIL and its agonistic antibodies as anticancer treatments selectively killed cancerous cells[94]. Despite promising results in preclinical studies, TRAIL-based therapies carry several disadvantages.

A randomized phase II study showed that a dual DR4 and DR5 agonist called dulanermin did not enhance the response of NSCLC patients to standard chemotherapy (paclitaxel, carboplatin, and bevacizumab)[95]. In a phase II study, the combination of mapatumumab with carboplatin and paclitaxel in advanced NSCLC patients showed no clinical benefit in unselected patients. No improvements were seen in disease control rates, overall survival, or median progression-free survival upon adding mapatumumab[96]. Tigatuzumab, a TRAIL-R2 agonist and a humanized monoclonal antibody with sorafenib, was tested in patients with advanced hepatocellular carcinoma in a phase II randomized study of safety and tolerability. Tigatuzumab with sorafenib (compared with sorafenib alone) in individuals with advanced hepatocellular cancer did not fulfill its primary efficacy endpoint, i.e., time-to-progression, although tigatuzumab with sorafenib is well tolerated in hepatocellular carcinoma[97]. Tigatuzumab did not increase the effectiveness of carboplatin/paclitaxel in patients with advanced NSCLC[98]. In a phase II clinical trial, Tigatuzumab with nanoparticle-albumin-bound paclitaxel appeared to enhance apoptosis in patients with triple-negative breast cancer[99]. Conatumumab, a fully human monoclonal IgG1 antibody that activates DR5 in combination with 5-fluorouracil, leucovorin, oxaliplatin (mFOLFOX6) and bevacizumab (bev), was tested for safety, tolerability, and efficacy in previously-untreated metastatic colorectal cancer patients. In the primary treatment of metastatic colorectal cancer patients, conatumumab with mFOLFOX6/bev showed no better efficacy than the same chemotherapy with a placebo[100].

The limited therapeutic potential is due to TRAIL resistance in several cancers[101]. The development of nanotechnology appears to be a promising strategy with several nano-based formulations of TRAIL and agonistic antibodies that help increase the circulating half-life and biodistribution of TRAIL in addition to its improved targeted delivery to tumor tissue.

NANOPARTICLE-BASED DRUG AND GENE DELIVERY SYSTEMS

Nanoparticles (NPs), also known as ultrafine particles, are a wide class of materials with sizes ranging between 1 to 100 nanometers. At present, a wide variety of nanoparticles serve as drug and gene delivery systems due to improved permeability and retention effect, increased stability and biocompatibility, and precision targeting[102]. Nanoparticles can be classified into many types, one among them being organic and inorganic nanoparticles based on the addition of organic and inorganic elements, respectively [Figure 4]. There is another type of nanoparticles, the hybrid nanoparticles, the conjugates of organic and/or inorganic materials. These hybrid nanoparticles help to overcome the drawbacks of the conventional nanoparticulate delivery systems, such as low water solubility, non-specific targeting, and poor therapeutic outcomes[103].In the below sections, we will give an overview of nanoparticle-based TRAIL therapy in different cancers. Nanoparticle formulations were tested in delivering recombinant TRAIL protein along with the TRAIL gene and the TRAIL receptor agonists in various models.

Table 1. TRAIL-R agonists/recombinant TRAIL in clinical trials

TRAIL-R agonists/ recombinant TRAIL	Mechanism	Cancer type	Clinical trials	References
AMG 951 (Dulanermin) (recombinant human TRAIL)	DR4 and DR5 activation	NSCLC	Phase-II	NCT00508625[80]
AMG 951 (Dulanermin) (recombinant human TRAIL)	DR4 and DR5 activation	Metastatic colorectal carcinoma	Phase-I	NCT00873756[81]
AMG 951 (Dulanermin)(recombinant human TRAIL)	DR4 and DR5 activation	Advanced non-small-cell lung cancer	Phase-III	NCT03083743[82]
HGS-ETR1 (Mapatumumab) (TRAIL-R1 agonist)	Activation of caspase-8, -9, -3, Bid, cleavage of PARP	Multiple myeloma	Phase-II	NCT00315757[83]
HGS-ETR1 (Mapatumumab) (TRAIL-R1 agonist)	Activation of caspase-8, -9, -3, Bid, cleavage of PARP	Hepatocellular carcinoma	Phase-II	NCT01258608[84]
HGS-ETR1 (Mapatumumab) (TRAIL-R1 agonist)	Caspase-8 activation, -9, -3, Bid, cleavage of PARP	Advanced NSCLC	Phase-II	NCT00583830[85]
HGS-ETR1 (TRAIL-R1 agonist)	Caspase-8 activation, -9, -3, Bid, cleavage of PARP	Relapsed/refractory NHL	Phase-II	NCT00094848[86]
CS-1008 (Tigatuzumab) (TRAIL-R2 agonist)	Antibody-dependent cell cytotoxicity	Metastatic or unresectable NSCLC	Phase-II	NCT00991796[87]
CS-1008 (Tigatuzumab) (TRAIL-R2 agonist)	Antibody-dependent cell cytotoxicity	Pancreatic cancer	Phase-II	NCT00521404[88]
CS-1008 (Tigatuzumab) (TRAIL-R2 agonist)	Antibody-dependent cell cytotoxicity	Metastatic triple-negative breast cancer	Phase-II	NCT01307891[89]
CS-1008 (Tigatuzumab) (TRAIL-R2 agonist)	Antibody-dependent cell cytotoxicity	Advanced hepatocellular carcinoma, Hepatic cancer, Liver cancer, Liver neoplasms	Phase-II	NCT01033240[90]
AMG 655 (Conatumumab) (TRAIL-R2 agonist)	DR5 activation	Hodgkin's lymphoma, Non-Hodgkin's lymphoma, LowGrade lymphoma, Lymphoma, Diffuse large cell lymphoma, Mantle cell lymphoma	Phase-I	NCT00791011[91]
AMG 655 (Conatumumab) (TRAIL-R2 agonist)	DR5 activation	Metastatic pancreatic cancer	Phase-Ib/II	NCT00630552[92]
AMG 655 (Conatumumab) (TRAIL-R2 agonist)	DR5 activation	Metastatic colorectal cancer	Phase-Ib/II	NCT00625651[93]

Nanoparticle-mediated TRAIL drug delivery

The various nanoparticles formulated to deliver TRAIL protein are discussed in detail below and Table 2.

Carbon-based nanoparticles

Carbon nanotubes, particularly single-wall carbon nanotubes (SWCNTs), are used in many physical and medicinal applications owing to their substantial flexibility, high mechanical resilience, and hydrophobicity. SWCNTs quickly diffuse in an aqueous medium. TRAIL-based SWCNT nanovectors showed greater efficiency than TRAIL alone in the induction of cancer cell death. Nanovectorization of TRAIL entails tagging TRAIL to SWCNTs to mimic membrane-bound TRAIL. His-tagged TRAIL was fused to SWCNT-pyrene-butyric acid N-hydroxysuccimide ester (PSE)-polyethylene glycol (PEG) to produce functional nanoparticles. The pro-apoptotic potential of these SWCNT-PSE-PEG-TRAILs (also called NPTs) is increased by almost 20-fold in various human tumor cell lines (HCT116- colon adenocarcinoma cells, H1703-squamous NSCLC cells, HepG2-hepatocarcinoma cells, and HUH-hepatoblastoma cells) due to increased levels of caspase-8 and enhanced DISC formation[104].

Another form of carbon-based nanoparticles is made from graphene, an allotrope of carbon. A graphene-based co-delivery nanosystem composed of graphene oxide, a PEG linker, and a furin-cleaved peptide

Table 2. Nanoparticle-based formulations of TRAIL protein for the treatment of various cancers

Nanoparticle type	Cancer type	Mechanisms to enhance TRAIL sensitivity	Reference
Single-walled carbon nanotubes	Colon adenocarcinoma, Squamous NSCLC, hepatocarcinoma, hepatoblastoma	Increased caspase-8 levels and enhanced DISC formation	[104]
Graphene nanosystem with doxorubicin	Lung & colon adenocarcinomas	Increased death receptor expression	[105]
Graphene quantum dots	Colon cancer	Increased pro-caspase-8 activation	[107]
Nanogold particles	Non-small cell lung cancer	Increased DR5 expression levels	[108]
Gold nanoparticles	Non-small cell lung cancer	Increased Drp1 recruitment to the mitochondria; Dysfunctioning of mitochondria	[109]
Silver-cysteine particles coated with polyethylene glycol	Colon cancer	Increased Bax and cleaved PARP; Decreased Bcl2 protein	[110]
Silver nanoparticles	Glioblastoma	Activation of death receptors; Increased caspase function	[111]
Double-edged lipid nanoparticles with doxorubicin	Hematological & epithelial carcinoma	Downregulation of FLIP and XIAP	[112]
LUV-TRAIL with doxorubicin	Breast cancer	Enhanced caspase-8 activation	[113]
LUV-TRAIL	Histiocytic lymphoma	Increased Bid, cleaved PARP, caspase-8, -3 & -10 levels; Enhanced DISC recruitment	[114]
LUV-type liposomes	Non-small cell lung cancer	Activation of caspase-8 & -3	[115]
LUV-TRAIL	Colon cancer	Increased caspase levels	[116]
Lipid nanoparticles	Hepatic fibrosis	Increased pro-apoptotic levels; Decreased uPA levels	[117]
Lumazine synthase protein cage nanoparticles	Epidermoid cancer	Intrinsic & extrinsic pathway activation	[118]
Ferumoxytol comprised of iron oxide nanoparticles	Colorectal cancer	Upregulation of DR5 levels; Overexpressed cleaved PARP	[120]
Magnetic ferric oxide nanoparticles	Glioma	Increased cleaved caspase-3 & cleaved PARP levels Increased pro-apoptotic potential	[121]
Maghemite nanoparticles	Breast & lung cancers	Increased pro-apoptotic potential Activation of caspase-3; Downregulation of Bcl-2 &BclxL; Upregulation of Bax& Bad; Increased death receptor levels	[122]
Iron oxide magnetic nanoparticles with actein	Non-small cell lung cancer	Activation of caspase-3; Downregulation of Bcl-2 &BclxL; Upregulation of Bax& Bad; Increased death receptor levels Increased pro-apoptotic potential	[123]
Iron oxide nanoclusters	Breast cancer	Increased pro-apoptotic potential Enhanced caspase-3 and -8 levels	[124]
SPION/TRAIL nanocomplex hydrogels	Glioblastoma	Enhanced caspase-3 and -8 levels Decreased survivin expression	[128]
NCL-240-loaded polymeric micelles	Ovarian cancer	Decreased survivin expression Increased caspase-3 & -7 levels; Decreased surviving & Bcl-2 levels	[129]
PEI-PLGA nanoparticle	Breast cancer	Increased caspase-3 & -7 levels; Decreased surviving & Bcl-2 levels Increased caspase-8 levels	[130]
Polymeric nanoparticles	Glioblastoma	Increased caspase-8 levels Increased caspase activity	[131]
Micellar nanoparticle with doxorubicin	Colorectal cancer	Increased caspase activity Increased caspase-3 &cleaved PARP levels	[132]
PCEC nanoparticles with curcumin	Colon cancer	Increased caspase-3 & cleaved PARP levels	[135]

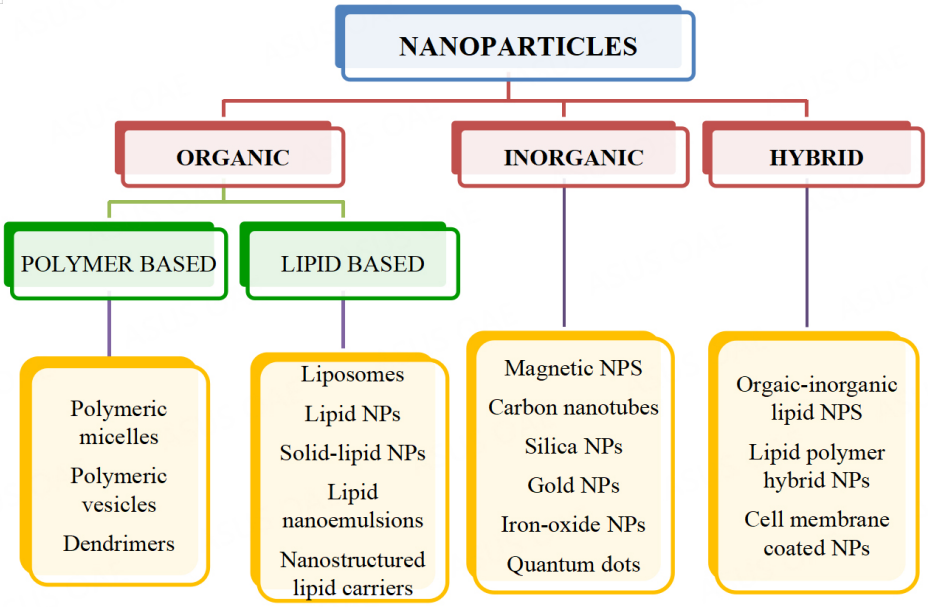

Figure 4. Types of Nanoparticles. Nanoparticles are classified based on the material used to prepare the various particles, organic, inorganic or hybrid. Different kinds of these formulations are represented here. NPs: Nanoparticles.

encapsulated with TRAIL and doxorubicin (Dox) resulted in the efficient release of TRAIL and Dox to their sites of action by digesting the peptide linker by furin with the increased expression of death receptors in lung and colon adenocarcinoma cells[105].

Docking studies of TRAIL nanovectorization were performed using graphene nanoflakes as a potential cargo for TRAIL. These studies showed that when adsorbed on graphene, TRAIL self-assembling and TRAIL affinities enhanced efficacy towards the targeted cancer cell. TRAIL bound to DR4 and DR5 more effectively when transported by graphene nanoflakes[106]. The synthesis of a novel nanohybrid system, sTRAIL (TRAIL fused with crystalline bacterial cell surface layer (S layer) protein), combined with graphene quantum dots or GQDs elevated the functional stability of TRAIL by improving pro-caspase-8 activation and mitochondria-dependent cell death in doxorubicin-pretreated human colon cancer cells (HT-29) when compared to S-TRAIL alone. The GQDs appeared to offer a surface for the S-TRAIL to self-assemble, facilitating TRAIL monomer oligomerization and enhancing apoptosis[107].

Gold nanoparticles

Nanogold-TRAIL complexes are nanogold coated with TRAIL protein. In the NSCLC microenvironment, M2 macrophages exhibit anti-inflammatory and pro-tumorigenic effects, while M1 macrophages exhibit antitumorigenic effects. Elimination of the M2 macrophages aids tumor destruction; hence, these M2 macrophages serve as potential targets. *In vitro* targeting M2 macrophages (derived from THP-1 monocytes) with nanogold-TRAIL particles led to 30-fold higher cytotoxicity. This enhanced cytotoxicity is attributed to increased surface-level DR5 expression and changes in the O-glycosylation pattern of the death receptor[108]. AuNPs (other nanoparticles made of gold) enhanced TRAIL sensitivity through dynamin-related protein 1 (Drp1)-mediated apoptotic and autophagic mitochondrial fission in NSCLC cells. AuNPs with TRAIL exhibited greater potency to promote apoptosis in NSCLC cells than TRAIL alone due to a rise in mitochondrial recruitment of Drp1 that led to dysfunctioning of mitochondria and induction of autophagy[109].

Silver nanoparticles

Experiments with silver-cysteine nanoparticles and TRAIL-conjugated silver nanoparticles coated with PEG (AgCTP NPs) showed that AgCTPNPs exhibited apoptotic effects in HT-29 colon cancer cell line in vitro with substantial differences in the expression levels of Bax, Bcl-2, and cleaved PARP protein[110]. TRAIL-conjugated AgNPs (TRAIL-AgNPs) induced death receptor activation in T98 G TRAIL-Resistant (TR) glioblastoma cells. The combination of TRAIL to silver nanoparticles (AgNPs) increased the functioning of caspases in TR glioblastoma cells, but TRAIL and AgNPs alone did not. Moreover, TRAIL-AgNPs-treated TR cells exhibited lower CHK1 expression than TRAIL-treated cells[111].

Lipid-based nanoparticles

Membrane-bound TRAIL in lipid nanoparticles, also known as large unilamellar vesicles (LUV)-TRAIL showed increased cytotoxic activity *in vitro* compared to soluble recombinant TRAIL in human sarcoma cells. LUV-TRAIL and anticancer drugs showed increased cytotoxicity against human sarcoma cells. LUV-TRAIL combined with flavopiridol (FVP) reduced human sarcoma cells' long-term clonogenic survival and triggered apoptosis by downregulating FLIP and XIAP and activating caspases, compared to soluble TRAIL (sTRAIL) alone[112]. LUV-TRAIL loaded with doxorubicin (Dox) in the liposomal lumen, known as LUVDOX-TRAIL, improved cytotoxic potential by enhanced caspase-8 activation in the tumor xenograft model of breast cancer cells[113].

LUV with tethered recombinant TRAIL (rTRAIL) was prepared and tested against U937 histiocytic lymphoma cells. LUV-TRAIL-induced cell death on U937 cells by causing cleavage of caspases faster than sTRAIL. Levels of Bid, cleaved PARP-1, caspase-8, -3, and -10 increased and enhanced recruitment of DISC upon treatment with LUV-TRAIL[114].

LUV-type liposomes coated with human sTRAIL were also used to treat NSCLC. Treatment of A549 cells with LUV-TRAIL activated caspases-8 and -3, compared with sTRAIL. LUV-TRAIL combined with antitumor medications such as CDK inhibitors, FVP, and SNS-032 further sensitized A549 NSCLC cells to LUV-TRAIL-induced apoptosis[115]. In HCT-116 and HT29 colon cancer cells, DR5 was more effectively activated by TRAIL-coated lipid nanoparticles (LUV-TRAIL) than by sTRAIL. When cells were exposed to various types of TRAIL, the pan-caspase inhibitor Z-VAD-FMK completely blocked cell death, suggesting that LUV-TRAIL-induced cell death is a caspase-dependent mechanism[116]. Lipid nanoparticles embedded with recombinant human TRAIL gave rise to significant apoptosis in hepatic fibrosis. Levels of the pro-apoptotic proteins were highly expressed, and the anti-apoptotic protein (uPA) levels were significantly decreased by a self-made drug carrier pPB-SSL with rhTRAIL protein[117].

Lumazine nanoparticles

A multiple ligand-displaying nanoplatform, lumazine synthase protein cage nanoparticle isolated from Aquifexaeolicus (AaLS) with TRAIL and EGFR binding affibody (EGFRAfb) via a SpyTag/SpyCatcher protein-ligation system (to form AaLS/TRAIL/EGFRAfb) exhibited TRAIL-mediated apoptosis in TRAIL-resistant and EGFR-overexpressing A431 epidermoid cancer cells *in vitro* through synergistic activation of the extrinsic and intrinsic apoptotic pathways[118].

Albumin nanoparticles

Albumin-bound NP technology was modified slightly to create TRAIL/Dox human serum albumin (HSA)-NPs, loaded with Dox and TRAIL nanoparticles. Using HCT116 colon cancer cells, the synergistic cytotoxicity and apoptotic activity of TRAIL/Dox HSA-NPs were assessed. Results from tumor tissues demonstrated that TRAIL/Dox HSA-NPs significantly increased apoptosis, while Dox HSA-NPs did so at a slightly lower level[119].

Magnetic/Iron oxide nanoparticles

Reactive oxygen species play critical roles in TRAIL-induced apoptosis. Iron oxide nanoparticles (iron oxide NPs) with ferumoxytol as its primary component spontaneously unite to form nanocomplexes with TRAIL/Apo-2L (NanoTRAIL). An investigation of the cell viability of HCT-116 (TRAIL-sensitive), SW-480 (TRAIL-intermediately resistant), and HT-29 (TRAIL-resistant) after treatments with saline, Apo-2L, iron oxide NPs, and NanoTRAIL resulted in a more modest TRAIL/Apo-2L response in HT29 than with other cell lines; there was a more significant anti-colorectal cancer tumor effect than with TRAIL/Apo-2L treatment alone in HT-29 cells with the upregulation of DR5 expression increased TRAIL/Apo-2L sensitivity and significant apoptosis. TRAIL sensitivity in the TRAIL/Apo-2L-resistant HT29 cells was due to activating JNK and increasing the expression of cleaved PARP[120].

Treating U251 cell-derived glioma xenografts with TRAIL conjugated to magnetic ferric oxide nanoparticles resulted in increased apoptosis, decreased tumor volume, and more prolonged survival. Conjugation of TRAIL to NP showed higher apoptosis levels than free recombinant TRAIL by increasing caspase-3, -8, and cleaved PARP levels[121]. Maghemite nanoparticles coated with TRAIL protein (CO-TRAIL@NPs-NH and NH-TRAIL@NPs-CO) enhanced apoptosis in human breast (MDA-MB-231) and lung (H1703) carcinoma cell lines compared to free TRAIL by increasing the pro-apoptotic potential[122].

Iron oxide (Fe_3O_4) magnetic nanoparticles combined with actein, a triterpene glycoside isolated from the rhizomes of Cimicifuga foetida, contributed to apoptosis in NSCLC. The induction of apoptosis in NSCLC cells resulted in the stimulation of the caspase-3 signaling pathway, which was identified by a decrease in levels of the anti-apoptotic proteins Bcl2 and Bcl-xL, increase in the pro-apoptotic signals Bax and Bad, and elevated levels of death receptors[123]. Interestingly, iron oxide nanoclusters (NCs) exhibited a synergistic effect with the TRAIL receptor. The engraftment of TRAIL onto NCs increased pro-apoptotic potential via nanoparticle-mediated magnetic hyperthermia (MHT) or photothermia in MDA-MB-231 wild-type and TRAIL receptor-deficient cells[124].

When compared to free recombinant TRAIL, magnetic ferric oxide nanoparticles enhanced apoptosis activity against several human glioma cells. There were NP-TRAIL molecules at the tumor site and a notable increase in glioma cell apoptosis in U251-derived glioma xenografts, which were examined for their effects on programmed cell death, tumor volume, survival, rhodamine-tagged NPs, and xenografts[125].

Polymer-based nanoparticles

Elastin-like polypeptides (ELPs) are potential thermosensitive biopolymers and polymeric drug delivery systems. The ability to induce apoptosis was three times greater when RGD-TRAIL was expressed coupled with ELPs than when RGD-TRAIL was expressed alone. When a single dose of the RGD-TRAIL-ELP nanoparticle was administered intraperitoneally, the COLO-205 tumor xenograft model showed nearly total tumor shrinkage. Protein electrophoresis revealed a 3.4-fold increase in the RGD-TRAIL-ELP trimer content than RGD-TRAIL[126]. P(RGD) proteinoids (where RGD is a tripeptide) comprising arginine, glycine, and aspartic acid, and proteinoid nanocapsules (NCs) were synthesized for targeted tumor therapy. TRAIL-P(RGD) nanocapsules induced cytotoxicity in CAOV-3 ovarian cancer cells more so than hollow P(RGD) nanocapsules which are atoxic in CAOV-3 cells. Dox-encapsulated P(RGD) nanocapsules also showed significant cytotoxicity in CAOV-3 cells[127].

SPION/TRAIL nanocomplex hydrogels, an MHT-mediated TRAIL release system with positively-charged TRAIL and hydrophobic superparamagnetic iron oxide nanoparticles (SPIONs) complexed with negatively-charged poly(organophosphazene) polymers via ionic and hydrophobic interactions, is used for combination therapy for multiple mild MHT and simultaneously MHT-induced TRAIL release. Hyperthermia restored the sensitivity of intrinsic TRAIL-resistant human glioblastoma (U87 MG) cancer cells and enhanced TRAIL-induced apoptosis by activating caspases-3 and -8[128].

A multifunctional nanoparticle system with a combination of a pro-apoptotic drug (NCL-240), TRAIL, and anti-survivin siRNA was used to test anticancer effects in various cancer cells. The NCL-240-loaded polymeric micelles (mNCL-240) and the combination of NCL-240-loaded, TRAIL-conjugated polymeric micelles (mNCL-240-loaded/TRAIL) showed significantly more cytotoxicity than single-drug formulations in all tested cell lines. Treating A2780 and U-87 MG cells with transferrin polymer micelles (Tf PM) improved the cytotoxic activity of NCL240 formulations, especially at higher doses. It was observed that mSurvivin (anti-survivin siRNA-S-S-PE mixed micelles) significantly downregulated survivin expression in ovarian cancer cells, A2780, compared to free anti-survivin siRNA and scrambled siRNA[129].

Hyaluronic acid (HA)-decorated polyethylenimine-poly(D,L-lactide-co-glycolide) (PEI-PLGA) nanoparticle (NP) (also known as HA/PPNP) with gambogic acid (GA) and TRAIL plasmid (pTRAIL) showed synergistic effects against breast cancer cells. Increased caspases-3 and -7 were observed in MDA-MB-231 cells treated with GA-HA/PPNPs, pTRAIL-HA/PPNPs, and GA/pTRAIL-HA/PPNPs by 2.39-, 1.18-, and 4.55-fold, respectively, after treatment of cells for 24 h compared to HA/PPNPs alone. Increased caspase-8 levels were also observed with pTRAIL-HA/PPNPs and GA/pTRAIL-HA/PPNPs by 1.47- and 1.55-fold, respectively, compared to untreated controls. Cells treated with GA/pTRAIL-HA/PPNPs showed the lowest expression levels of survivin and Bcl-2 anti-apoptotic proteins[130]. The polymeric nanoparticle-engineered human adipose-derived stem cells (hADSCs) overexpressing TRAIL served as drug-delivery vehicles for targeting and eliminating glioblastoma multiforme (GBM) cells *in vivo*. The co-culturing of plasmid human TRAIL-laden nanoparticles (NPs/pTRAIL)-engineered hADSCs with patient-derived malignant glioma xenograft cells (D-270 MG) resulted in significant apoptosis and death in glioma cells (compared to controls) through an increase in levels of caspase-8 and the caspase cascade[131]. The delivery of micellar nanoparticles self-assembled from a biodegradable cationic copolymer P(MDS-co-CES) with TRAIL and Dox showed synergistic cytotoxic effects in TRAIL-resistant SW480 colorectal cancer cells. In cells treated with nanoparticles, in the presence of ZVAD-FMK, a pan-caspase inhibitor gave rise to TRAIL sensitivity, suggesting that apoptosis depends on caspase activity[132].

N-{[(2-hydroxy-5-nitrophenyl)amino] carbonothioyl}-3, 5-dimethylbenzamide (DM-PIT-1)-loaded polyethylene glycol and phosphatidylethanolamine (PEG-PE) micelles modified with TRAIL were used against TRAIL-resistant U87MG cells. In contrast to PEG-PE micelles loaded with DM-PIT-1, drug-free PEG-PE micelles modified with TRAIL are not cytotoxic for U87MG cells. The synergistic effect and the significantly increased cell death were caused by the extra attachment of TRAIL to drug-loaded micelles. Compared to groups treated with typical DM-PIT-1-loaded micelles, all groups treated with TRAIL-modified DM-PIT-1-loaded PEG-PE micelles significantly reduced viability in U87MG cells[133]. PEGylated heparin, poly-L-lysine nanoparticles with TRAIL called TRAIL-PEG-NPs, had apparent apoptotic effects when administered *in vitro* and *in vivo*. TRAIL-PEG-NPs induced time-dependent apoptotic cell death and efficiently suppressed mean tumor growth with mean tumor growth inhibition[134]. The hydrophobic drug curcumin (Cur) encapsulated into the inner core of biodegradable poly (e-caprolactone)-poly (ethylene glycol)-poly (e-caprolactone) nanoparticles with TRAIL protein (TRAIL-Cur-NPs) was evaluated for their efficacy to treat HCT116 human colon carcinoma cells. Compared to the free TRAIL + Cur group, protein

expression of cleaved caspase-3 and cleaved PARP was slightly higher in the TRAIL-Cur-NPs group. The overexpression of DR4 and DR5 synergistically increased the anticancer efficacy of TRAIL and Cur[135]. TRAIL-modified and cabazitaxel (CTX)-loaded polymer micelles (TRAIL-M-CTX) were synthesized to test the anticancer efficacy against TRAIL-resistant MCF7 breast cancer cells. Synergistic effects of apoptosis were observed in MCF7 cells treated with TRAIL-loaded nanoparticles than in CTX and TRAIL alone treated cells[136].

Nanoparticle-mediated TRAIL gene delivery

Several studies described the use of TRAIL protein-based nanoparticles. These trials have been successful but are limited by the relatively short half-life of the ligand, targeted delivery to the tumor cells, and proper presentability to the death receptors on the membrane. Hence, novel approaches have been attempted, one of which is delivering the TRAIL gene to the tumor cells instead of the protein[137]. This section summarizes studies of TRAIL gene-based nanoparticles [Table 3].

Magnetic/Iron oxide nanoparticles

The TRAIL gene combined with polyethyleneimine-coated magnetic iron oxide nanoparticles (also known as polyMAG-1000) triggered significant apoptosis in MCF-7 breast cancer cells[138].

The transfection of the TRAIL gene with polyethyleneamine-coated polyMAG-1000 combined with anticancer drug cisplatin/cis-diamminedichloroplatinum(II) (CDDP) triggered apoptosis in A2780/DDP ovarian cancer cells. Compared to TRAIL gene transfection or CDDP therapy alone, more apoptosis was seen in the presence of the medication. Cytochrome C release and the caspase-9 cleavage pathway were linked to the triggering of apoptosis in A2780/DDP cells[139].

GBM is among the most aggressive types of brain cancer. The successful delivery of TRAIL-encoded plasmid DNA into human T98G GBM cells with chitosan-polyethylene glycol-polyethyleneimine copolymer and CTX-coated iron oxide nanoparticles successfully induced a three-fold increase in apoptosis compared to control cells[24].

A nanosystem for magnetofection was used to create magnetic nanoparticles carrying the TRAIL gene and chitosan. In a melanoma pulmonary metastatic mouse model, the mouse melanoma cell line B16F10 was injected, and the mice were monitored for metastatic tumor formation in the lungs. These animals were then given a systemic injection of the abovementioned nanoparticles through the tail vein. Nanoparticles reaching the lungs were activated to express the TRAIL gene by an external magnetic field applied at the rib cage. There was a significant increase in tumor cell death and suppression of the development of metastatic melanoma[140]. In another study, the authors constructed a Fe_3O_4-PEI-plasmid complex (FPP) in which positively-charged PEI altered iron oxide nanoparticles to enable them to carry the negatively-charged plasmid pACTERT expressing TRAIL. *In vitro* and *in vivo* apoptosis of SACC-83 adenocystic carcinoma cells was successfully induced by FPP-mediated TRAIL gene transfer.

A substantial increase in apoptosis was observed in transfected SACC-83 cells. An analysis of anticancer properties of the magnetic complex-TRAIL in mice showed significantly smaller tumor size after transfection than the control groups[141].

Gold nanoparticles

The combination of the secretable trimeric Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (stTRAIL) gene and PEI-capped gold nanoparticles (AuNPs) increased the inhibition of cell proliferation

Table 3. Nanoparticle-based formulations of TRAIL gene for the treatment of various cancers

Nanoparticle type	Cancer type	Mechanisms to enhance TRAIL sensitivity	Reference
PEI-coated superparamagentic iron oxide nanoparticles with cisplatin	Ovarian cancer	Cytochrome-C release and caspase-9 cleavage	[139]
Chitosan magnetic nanoparticles	Melanoma	Activation of caspase-3	[140]
PEI-capped gold nanoparticles	Hepatoma	Increased stTRAIL's mRNA and protein levels; Increased caspase-8 and -3 levels	[142]
Zein nanoparticles	Liver cancer	Increased p53 expression; Decreased MMP-2 expression	[143]
NIR light absorbing conjugated polymer nanoparticles	Breast cancer	Increased caspase-8 cleavage	[144]
Poly(beta-amino ester) nanoparticles	Liver cancer	Increased DR5 expression	[145]
Triazine modified PAMAM dendrimer	Osteosarcoma	Increased caspase-3, -7, cleaved PARP levels	[149]
RRPHC polymeric nanoparticles	Melanoma	Increased caspase-9 & -3 levels	[150]
Poly(beta-amino ester) nanoparticles	Lung cancer	Increased DR4 & DR5 expression levels	[151]
RRPHC nanoparticles	Colorectal cancer	Increased cleaved caspase-9 & -3 levels Increased levels of cleaved caspase-3 & -9 levels	[152]
PEI-R8 heparin nanogel	Colon cancer	Increased levels of cleaved caspase-3 & -9 levels	[153]
γ-PGA/Moβ-CD-SSPEI pDNA nanocomplexes	Colon & cervical cancers	Increased DR5 & cleaved PARP levels	[154]
PEI-CD/Ad-Dox/pDNA supramolecular nanoparticles	Ovarian cancer	Increased apoptotic protein levels	[155]
LCPP nanoparticles	Hepatocellular carcinoma	Increased DR5 expression phosphorylation	[157]
Lipid-Protamine-DNA nanoparticles		Decreased mTOR phosphorylation; Increased AMPK-alpha	[158]
Cationic albumin-pegylated nanoparticles	Glioma	Increased cleaved p17 fragment; Increased caspase-3 levels	[160]

and promoted apoptosis in heat-shocked hepatoma cells; there was increases in the levels of stTRAIL mRNA and protein and caspases-8 and -3[142].

Zein nanoparticles

Zein is a protein derived from maize that is prolamine-rich and is used as a nanocarrier because of its distinct structure, physicochemical characteristics, and self-assembly process. The growth rate and cell viability of HepG2 liver tumor cells treated with TRAIL-loaded zein nanoparticles (ZNPs) were lower than untreated cells in a concentration-dependent manner. Treatment with TRAIL genes packaged into ZNPs resulted in less MMP-2 expression in liver homogenates than untreated ones[143].

Nano-polymers

Conjugated polymer nanoparticles (CPNs) that absorb near-infrared light were developed to remotely control and induce TRAIL-mediated apoptotic signaling to boost apoptosis in TRAIL-resistant cancer cells[144]. In response to heat shock, the promoter of a heat shock protein initiated transcription of the TRAIL gene; as a result, breast cancer cells expressed the TRAIL protein, activating the TRAIL-mediated death signaling pathway. The CPNs simultaneously produce W-7 (a calmodulin antagonist) which promotes caspase-8 cleavage and increases cancer cell death. *In vitro* and *in vivo* tests revealed that CPNs/W-7/p TRAIL has a considerable synergistic therapeutic effect on breast cancer and is barely toxic when exposed to near-infrared light. In HepG2 cancer cells, poly(beta-amino ester) nanoparticles (PBAE NPs), engineered with a cDNA sequence coding a secretable TRAIL protein or sTRAIL, initiated apoptosis by increased DR5 expression on the cell surface and a 40-fold increase in cell death, reprogramming liver cancer cells to produce TRAIL protein[145]. The combination of positively-charged polymer, PEI-modified Fe_3O_4 magnetic

nanoparticles, and negatively-charged pACTERT-EGFP through electrostatic interaction resulted in a new magnetic nanovector (pACTERT-TRAIL) with antitumor properties in cell lines and xenograft models of oral squamous cell carcinoma[146].

Polyamidoamine (PAMAM) dendrimers are distinctive nanostructures and are effective non-viral carriers because of their highly branched, three-dimensional structure with high loading capacity and lack of toxicity. Modifying G4 and G5 PAMAM dendrimers with alkyl carboxylate-PEG and cholesterol triggered apoptosis in colon cancer cells *in vitro* and *in vivo*. *In vivo*, there was a significantly higher therapeutic index of the plasmid TRAIL in modified PAMAM dendrimer (pTRAIL) than in unmodified PAMAM. Cells treated with the PAMAM G4 derivative coupled with alkyl-PEG and cholesterol (F5-G4) and TRAIL (F5-G4-TRAIL) and PAMAM G5 derivative with alkyl-PEG and cholesterol (F5-G5) and TRAIL (F5-G5-TRAIL) demonstrated 20.1% and 24.14%, of cell death, respectively[147].

The combination of D-α-tocopheryl polyethylene glycol 1000 succinate-b-poly (ε-caprolactone-ran-glycolide), also known as TPGS-b-(PCL-ran-PGA) (a biodegradable diblock copolymer), PEI, and TRAIL showed anticancer effects *in vitro* and *in vivo* in HeLa cells. SCID mice carrying HeLa tumor xenografts showed increased cytotoxicity when treated with nanoparticles loaded with TRAIL[148]. Triazine, a molecule with DNA binding capacity, modified on a PAMAM dendrimer tagged with green fluorescence protein and TRAIL reporter gene (G5-DAT66/pTRAIL complex), was constructed and treated against an osteosarcoma cell line (MG-63). Higher levels of apoptosis were observed in cells treated with dendrimer and TRAIL complexes than in cells treated with dendrimer or TRAIL alone, with increased levels of caspase-3, -7, and cleaved PARP[149]. The design of core-shell ternary systems consisting of fluorinated polymers (PFs) binding with a plasmid (pDNA) and a negatively-charged multifunctional RRPH (arginyl-glycyl-aspartic acid (RGD)-cell-penetrating peptide (R8)-PEG-sodium hyaluronate/(RGD-R8-PEG-HA) shell, also known as RRPH/PF/pDNA (RRPHC) ternary complex or HA (sodium hyaluronate) shell, also known as HA/PF/pDNA (HAC) ternary complex for the delivery of pUNO1-mTRAIL (mouse TRAIL) plasmid was successful in causing apoptosis in B16F10 mouse melanoma cell line. Transfection with the complexes containing mTRAIL significantly inhibited the growth of the B16F10 cells and induced elevated apoptosis effect with increased expression levels of pro-apoptotic proteins (cleaved caspase-9 and cleaved caspase-3). The RRPHC/mTRAIL complexes induced a comparable apoptosis effect higher than that of HAC/mTRAIL ternary complexes[150].

Several human cancer cell cultures can be transfected *in vitro* by DNA-containing polymeric nanoparticles based on PBAEs, leading to cell death. Recombinant human TRAIL (rhTRAIL) was not as successful as the PBAE/TRAIL-DNA therapy in killing H446 lung cancer cells, which also demonstrated high levels of DR4 protein expression, high levels of DR5, and low levels of DcR1 and DcR2 expression[151]. According to a study on the anticancer activity of the spontaneous nucleus-targeting core PF33/hTRAIL, HAC/hTRAIL, and RRPHC/hTRAIL complexes on HCT116 colorectal cancer cells, the TRAIL protein was significantly more highly expressed in the groups treated with PF33/hTRAIL and RRPHC/hTRAIL than in the groups treated with HAC/hTRAIL. The expression of cleaved caspase-9 and -3 proteins showed similar results, suggesting that the hTRAIL is responsible for apoptotic action[152]. A novel PEI-RRRRRRRR (R8)-heparin (HPR) nanogel with a plasmid containing human TRAIL gene (HPR/phTRAIL) showed higher levels of cleaved caspase-3 and -9 in HCT-116 colon cancer cells. HPR nanogel delivered phTRAIL into HCT-116 cells to express hTRAIL protein and significantly induced apoptosis[153].

Carbon-based nanoparticles

In HCT8/ADR and HeLa cells, the cytotoxic activity of pDNA nanocomplexes containing PGA/Mo-CD-SSPEI and redox-sensitive bioreducible PEI with plasmid TRAIL was assessed in the presence and absence of monensin. By increasing DR5 and cleaved PARP levels, the pTRAIL-induced apoptotic rate in HCT8/ADR and HeLa cells was greater than that of the control groups. These findings suggest that the RGD-PGA/Mo-CD-SSPEIpTRAIL and PGA/Mo-CD-SSPEIpTRAIL groups had significant levels of TRAIL protein expression in the tumors. The dual-targeting RGD-PGA surface coating provided the best cancer cell treatment efficacy[154].

Dox and TRAIL therapeutic gene-loaded PEI-CD/Ad-Dox/pDNA supramolecular nanoparticles (SNPs) were assayed for treating ovarian cancers. The PEI-CD/Ad-Dox SNP included in the pTRAIL prevented ovarian tumor growth *in vivo* and significantly increased the survival time of tumor-bearing mice. Free medication and plasmid-loaded PEI-CD/Ad-Dox were tested in an *in vitro* retention experiment on SKOV-3 ovarian tumors, with PEI-CD/Ad-Dox/pTRAIL exhibiting the highest levels of cytotoxicity. When different groups were compared, more apoptotic cells and more significant expression of apoptotic proteins were found in the SNP-treated group[155].

Lipid-based nanoparticles

The TRAIL gene carrying cationic nanoliposomes (pDsRedl-Cl-TRAIL/Lipofectamine 2000) transfected into dendritic cells induced apoptosis in LoVo colorectal cancerous cells *in vitro* and *in vivo*[156]. The synthesis of a tumor-targeted lipid/calcium/phosphate/protamine (LCPP) nanoparticle to deliver TRAIL pDNA into hepatocellular carcinoma (HCC) cells with an HCC-targeting peptide (SP94) gave rise to apoptosis. Due to the stimulation of calmodulin-dependent protein kinase II, SP1, and calcium signaling, SP94-LCPP NPs carrying TRAIL pDNA dramatically enhanced TRAIL expression and triggered TRAIL-mediated cytotoxicity in human (Hep3B, JHH-7) and murine (HCA-1) HCC cells. Compared to previous treatments, TRAIL pDNA supplied by SP94-LCPP NPs dramatically boosted the number of apoptotic cells in orthotopic HCA-1 tumors. Compared to the single treatments, the combination of sorafenib with NP-induced TRAIL production caused HCC cells to undergo significantly higher levels of apoptosis[157].

A novel lipid (1, 2-di-(9Z-octadecenoyl)-3-biguanide-propane (DOBP)) was encapsulated with TNF-related apoptosis-inducing ligand plasmids (TRAIL plasmids) into lipid-protamine-DNA nanoparticles (LPD NPs) for systemic gene delivery. Comparing 1, 2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP)-LPD-TRAIL nanoparticles to DOBP-LPD TRAIL nanoparticles, it was clear that the latter were significantly less effective at slowing tumor growth. In contrast to phosphate-buffered saline and Lipo-DOTAP, tumor tissues treated with metformin and Lipo-DOBP showed significantly greater levels of AMPK-alpha phosphorylation, while mTOR phosphorylation was downregulated[158].

The synthesis of another type of nanoparticles (rHDL/PEI-LA/pTRAIL) comprising a reconstituted high-density lipoprotein (rHDL), lauric acid-coupled polyethyleneimine (PEI-LA) as an amphipathic positively-charged polymer with pTRAIL was engineered into mesenchymal stem cells (MSCs) for the treatment of lung metastasis of melanoma. *In vitro* and *in vivo*, the rHDL-mediated TRAIL-engineered MSCs had a promising potential to target the B16F10 pulmonary melanoma metastases[159].

Albumin-based nanoparticles

TRAIL gene delivered with plasmid pORF-hTRAIL (pDNA), incorporated into cationic albumin-conjugated pegylated nanoparticles (CBSA-NP), was evaluated as a non-viral vector for gene therapy of gliomas; hTRAIL-mediated apoptosis was assessed by immunohistochemical analysis for active caspase-3

using an antibody that detected the cleaved p17 fragment at 14 days after intravenous administration of CBSA-NP-hTRAIL, which was confirmed by the presence of p17-positive tumor cells[160].

CONCLUSION

TRAIL is an apoptosis-inducing ligand that kills cancer cells without affecting normal cells. TRAIL binds to specific death receptors on the cell surface and triggers the apoptosis extrinsic and intrinsic pathways. The targeted killing of specific tumor cells makes it a promising molecule for cancer treatment. TRAIL receptor agonists designed to bind and activate death receptors in the absence of TRAIL proved promising. Interestingly, TRAIL failed in clinical trials due to its relatively short half-life, decreased protein stability, reduced bioavailability, and resistant mechanisms exhibited by cancer cells to evade apoptosis. For successful therapy, the bioavailability of TRAIL was enhanced by synthesizing soluble and stable peptides of TRAIL and encapsulating them in nano-formulations that aided the targeted delivery of stable, active TRAIL to tumor cells. Various organic, inorganic, and hybrid nanoparticles carrying the TRAIL protein, gene, or receptor agonists were tested in *in vitro* studies in several cancer cell lines. Nanoparticle-mediated TRAIL delivery significantly enhanced apoptosis by increasing death receptor clustering, DISC formation, and caspase activation. Combinatorial treatment of tumor cells with nano-TRAIL and other chemotherapeutic agents also sensitized the resistant cancer cells to TRAIL. These studies suggest that applying nanotechnology to TRAIL therapy is successful in cell lines and preclinical animal models. Further experimentation and standardization are required for the success of nano-formulations of TRAIL.

Insights are required to search for biomarkers that can mark cells as TRAIL-sensitive, novel methods to deliver TRAIL precisely, and the use of TRAIL combined with other medications/natural products to overcome resistance. One compelling approach is the application of TRAIL nanoparticles in cancer treatment for the delivery of TRAIL protein or gene to tumor cells.

DECLARATION

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Authors' contributions

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

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Not applicable.

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Cancer Drug Resistance

Original Article

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Targeting hormone-resistant breast cancer cells with docetaxel: a look inside the resistance

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Abstract

Aim: The study aims to analyze the effect of long-term incubation of ERα-positive MCF7 breast cancer cells with 4-hydroxytamoxifen (HT) on their sensitivity to tubulin polymerization inhibitor docetaxel.

Methods: The analysis of cell viability was performed by the MTT method. The expression of signaling proteins was analyzed by immunoblotting and flow cytometry. ERα activity was evaluated by gene reporter assay. To establish hormone-resistant subline MCF7, breast cancer cells were treated with 4-hydroxytamoxifen for 12 months.

Results: The developed MCF7/HT subline has lost sensitivity to 4-hydroxytamoxifen, and the resistance index was 2. Increased Akt activity (2.2-fold) and decreased ERα expression (1.5-fold) were revealed in MCF7/HT cells. The activity of the estrogen receptor α was reduced (1.5-fold) in MCF7/HT. Evaluation of class III β-tubulin expression (TUBB3), a marker associated with metastasis, revealed the following trends: higher expression of TUBB3 was detected in triple-negative breast cancer MDA-MB-231 cells compared to hormone-responsive MCF7 cells ($P < 0.05$). The lowest expression of TUBB3 was found in hormone-resistant MCF7/HT cells (MCF7/HT < MCF7 < MDA-MB-231, approximately 1:2:4). High TUBB3 expression strongly correlated with docetaxel resistance: IC_{50} value of docetaxel for MDA-MB-231 cells was greater than that for MCF7 cells, whereas resistant MCF7/HT cells

were the most sensitive to the drug. The accumulation of cleaved PARP (a 1.6-fold increase) and Bcl-2 downregulation (1.8-fold) were more pronounced in docetaxel-treated resistant cells ($P < 0.05$). The expression of cyclin D1 decreased (2.8-fold) only in resistant cells after 4 nM docetaxel treatment, while this marker was unchanged in parental MCF7 breast cancer cells.

Conclusion: Further development of taxane-based chemotherapy for hormone-resistant cancer looks highly promising, especially for cancers with low TUBB3 expression.

Keywords: Cancer, docetaxel, 4-hydroxytamoxifen, class III β-tubulin, resistance, breast cancer, estrogen receptors alpha

INTRODUCTION

Breast cancer is considered a major cause of death from cancer in women around the world[1]. Breast cancer has several various molecular subtypes, and more than 65%-70% of breast tumours are hormone receptor-positive (ERα+)[2]. ERα-positive cancers are initially driven by hormone activation of estrogen receptor α, which in turn, induces pro-proliferative/pro-oncogenic cascades[3]. This fact explains the effectiveness of therapies that target the hormone molecular pathway in ERα-positive breast cancer[4]. The first antiestrogen for ERα-positive breast cancer treatment was tamoxifen, a selective estrogen receptor modulator, inhibiting the activity of the estrogen receptor alpha (ERα)[5,6]. The mechanism of its action in breast cells is the competition with endogenous estrogens for binding estrogen receptor α; thus, tamoxifen inhibits the estrogen-driven, pro-proliferative transcription program in breast cancer cells[6,7] and also activates G-protein-coupled ER (GPER1)[8]. In some cases, long-term tamoxifen treatment leads to the development of resistance, cellular mechanisms of which are complex and not fully clear. They include regulation of ESR1 expression by epigenetic factors[9], mutations of ESR1[10], alternative splicing events[11], alterations in the hormone-binding domain[10], differential recruitment of coregulators[12], factors of the tumor microenvironment[13] and many others [14,15]. One of the major mechanisms of the development of hormone resistance is dysregulation of the PI3K/AKT/mTOR pathway that cross-talks with estrogen-mediated signaling[16]. Inhibition of the PI3K/AKT/mTOR pathway results in reduced cell proliferation and survival, but this activates compensatory mechanisms that confer resistance to inhibitors. In several studies, it was shown that activation of the Akt pathway was associated with tamoxifen resistance in breast cancer cells, poor prognosis and decreased relapse-free survival, and increased incidence of relapse with distant metastases[17-19].

βIII-tubulin is a well-known tubulin isotype. Monomers of α- and β-tubulin spontaneously assemble and polymerize to form the microtubules, cytoskeletal polymers involved in critical cellular processes such as mitosis, cell motility, and intracellular transport. Moreover, β-tubulins are GTPases as well and regulate the kinetics of microtubule assembly and disassembly[20]. βIII-tubulin is overexpressed in many tumours, including resistant tumours[21,22], and is regulated by hormones[23]. Docetaxel, a taxane, is an antimicrotubule agent effective in the treatment of patients with breast cancer[24]. Researchers are conducting extensive investigation of this drug to improve treatment efficacy and delivery selectivity[25-27]. The study aims to analyze the effect of long-term incubation of ERα-positive breast cancer cells with 4-hydroxytamoxifen on their sensitivity to a tubulin polymerization inhibitor docetaxel.

METHODS

Cell lines and compounds

The triple-negative MDA-MB-231 and hormone-dependent MCF7 breast cancer cell lines were purchased from ATCC collection. The cells were maintained in a standard DMEM medium (Gibco) supplemented

with 10% fetal bovine serum (FBS, HyClone) at 37 °C, 5% CO_2 and 80%-85% humidity (NuAire CO_2 incubator).

4-hydroxytamoxifen and docetaxel were purchased from Cayman Chemical Company; drug solutions were stored at -70 °C. MCF7/HT cell line was obtained by long-term (for 12 months) cultivation of parental MCF7 cells with antiestrogen 4-hydroxytamoxifen at a concentration of 5 μM. The verification of acquired hormone resistance in MCF7/HT was done by the MTT test.

The analysis of cell viability

The analysis of cell viability was performed by the MTT test[28] as described earlier in the work[29]. The cells were seeded at a density of 4×10^4 cells per well in 24-well plates (Corning) in 900 μL of the medium. The solutions of the tested compounds (4-hydroxytamoxifen, docetaxel) with different concentrations in 100 μL of the medium were added 24 h after the seeding. The cells were cultivated for 72 h, then the medium was removed, and the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) dissolved in the medium was added to the final concentration of 0.2 mg/mL to each well and incubated for 1 h. After that, the medium was removed, and MTT formazan purple crystals were dissolved in DMSO (300 μL per well). Absorbance was measured at 571 nm with a MultiScan reader (ThermoFisher). The half-maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism.

Assessment of ERα activity

MCF7 and MCF7/HT cells were seeded onto a 24-well plate containing a standard cell culture medium with a density of 1.7×10^5 cells/well. After 24 h, the cells were transfected with the ERE-LUC plasmid containing the luciferase gene under the ERα-dependent promoters[30], and co-transfected with β-galactosidase plasmid. The transfection was performed for 6 h at 37 °C using Lipofectamine 2000 in a medium containing 2% steroid-free serum, then the medium was replaced by a standard medium supplemented with 10% steroid-free serum. The activity of luciferase was assessed according to a Promega protocol using an Infinite M200-Pro, and β-galactosidase activity was assessed using colorimetric assay and MultiScan FC. The internal control values were used to normalize the luciferase/β-galactosidase activities. ERα activity was represented as the mean ± SD for the three independent experiments.

Immunoblotting and densitometry

Immunoblotting with modifications was performed as described earlier[31]. ERα, phospho-Akt, Akt, cleaved PARP, Bcl-2, Bad, phospho-ERKs, ERKs, phospho-S6K, S6K and cyclin D1 expression was evaluated using Cell Signalling Technology (CST) antibodies. Antibodies to α-tubulin (CST) were applied to normalize and control the loading of samples into a gel. Secondary antibodies to rabbit Ig conjugated with horseradish peroxidase were used for the detection (Jackson ImmunoResearch). ECL detection reagents for analysis were prepared according to Mruk and Cheng's protocol[32]. ImageQuant LAS 4000 imager (GE Healthcare) was used to detect signals. Densitometry for the tested proteins/α-tubulin ratio was carried out using ImageJ software.

Flow cytometry

TUBB3 expression was evaluated by immunofluorescence assay and flow cytometry[33]. The cell suspensions at a concentration of 4×10^5 cells/ml were incubated for 1.5 h with primary monoclonal anti-TUBB3 antibody (clone EP1569Y, Abcam, UK) at room temperature in the dark; the suspensions were then incubated for 1.5 h with secondary anti-rabbit antibody conjugated with DyLight®650 (ab98510, Abcam, UK) at + 4°C in the dark. The final dilution for both antibodies was 1:500. Cell fluorescence was measured using the Navios flow cytometer (Beckman Coulter, USA). Two indicators of βIII-tubulin expression were evaluated by FlowJo 10.0.8 (FlowJo, USA): the geometric mean of fluorescence (arb. units) and level of the

marker expression (number of specific fluorescent cells). The level of TUBB3 expression was calculated by the Kolmagorov-Smirnov test as the ratio of specifically fluorescent cells to the control cells incubated with secondary antibodies only. The distribution of cells according to fluorescence intensity was visualized using WinMDI 2.9 software.

Statistical analysis

All data are presented as mean values and standard deviation (mean ± std. deviation). Student's t-test (GraphPad Prism 9, USA) at $P < 0.05$ was considered to indicate a statistically significant result.

RESULTS

In vitro experiments are an important part of any study in biology and medicine. Their importance is particularly reinforced by modern standards of ethics in science. So, there is a need to analyze the relationship between the doses of a drug given to patients and the concentration of the compound in the culture medium. Are such doses comparable? We first analyzed data from experiments with hormones and their antagonists. The level of 17β-estradiol undergoes significant changes in premenopausal women; usually, the level of this hormone is between 30 and 400 pg/mL[34-37]. Thus, we cannot talk about an average level of 17β-estradiol in plasma; in experimental studies, most researchers apply a dose of 17β-estradiol that induces the expression of responsive genes, 10 nM or 2.7 ng/mL[38,39]. We see similar trends for antiestrogens, which are used as 17β-estradiol competitors (selective estrogen receptor modulators, SERMs). In the plasma of breast cancer patients who receive tamoxifen, from 391 to 484 ng/mL of the major metabolites of this drug (tamoxifen, N-desmethyltamoxifen, hydroxytamoxifen, endoxifen) were determined[40,41]. In *in vitro* experiments, the IC_{50} values of tamoxifen usually exceed the level of 500 ng/mL and were 1-20 μg/mL[42-44]. A slightly different situation is observed in the case of chemotherapeutics, in particular docetaxel. The plasma concentration of docetaxel reaches 3737 nM clinically[45], whereas in cell culture experiments, the IC_{50} values do not exceed 10 nM[46-48]. Thus, the hormonal drugs are used in higher doses in experiments, while docetaxel is applied in *in vitro* experiments at doses lower than those in the plasma of patients receiving drug treatment. The observations described are consistent with the duration of treatment; hormone therapy is prescribed for long courses (up to 10 years), whereas chemotherapy can be prescribed in short courses due to its high toxicity. In the *in vitro* study presented here, we started from the IC_{50} values for 4-hydroxytamoxifen and assessment of hormonal signaling in obtained resistant cells.

MCF7/HT was obtained by long-term cultivation of MCF7 breast cancer cells with 4-hydroxytamoxifen. The established MCF7/HT subline has lost sensitivity to 4-hydroxytamoxifen (HT), which was confirmed by the MTT test: the viability of MCF7/HT in the presence of higher concentrations of 4-hydroxytamoxifen compared to MCF7 indicates the developed resistance, the resistance index (IC_{50} of MCF7/HT divided by IC_{50} of MCF7) was 2 [Figure 1A].

It is known that the acquisition of tamoxifen resistance in MCF7 cells is accompanied by the impaired activation of the PI3K/Akt/*PTEN* pathway and down-regulation of ERα[17,49,50]. Following the literature data, we have revealed that Akt activity (p-Akt) in MCF7/HT was increased by 2.2-fold [Figure 1B and C]. Moreover, ERα expression was decreased by 1.5-fold.

To assess ERα activity, the cells were transfected with the plasmids containing the luciferase gene under the estrogen receptor α-dependent promoters (ERE-LUC). The activity of ERα was induced by its physiological ligand, 17β-estradiol[30,52]. As can be seen in Figure 2, the induced activity of the estrogen receptor α in MCF7/HT cells was decreased by 1.5-fold ($P < 0.05$) when compared to that in parental cells. This indicates a partial loss of hormonal dependence of the breast cancer cells after long-term incubation with 4-hydroxytamoxifen.

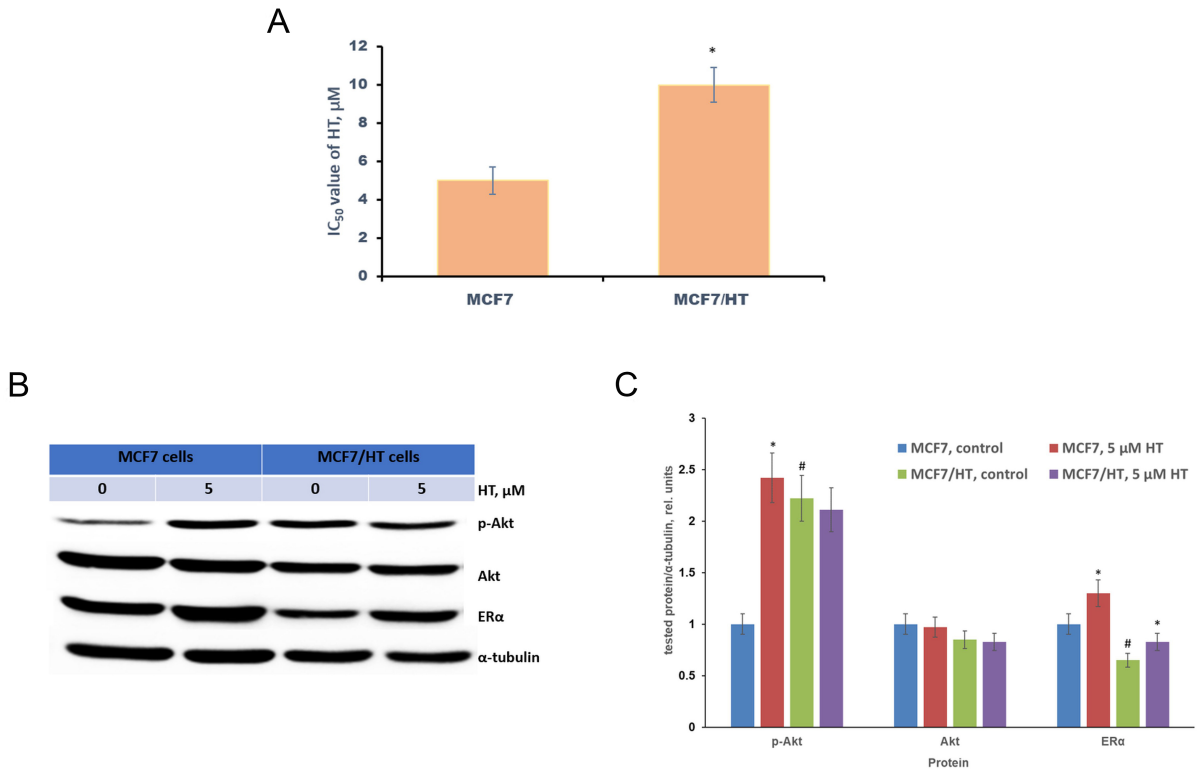

Figure 1. Characteristics of the obtained subline MCF7/HT. (A) - the viability of MCF7/HT and MCF7 cells in the presence of 4-hydroxytamoxifen (HT), **P* < 0.05; (B) - Representative immunoblotting images for sensitive MCF7 and resistant MCF/HT cells with antibodies against ERα, p-Akt, and Akt; the cells were treated with 5 μM HT for 24 h and then subjected to immunoblot analysis; (C) - Densitometry for immunoblotting data (*n* = 3) was performed using ImageJ software (Wayne Rasband, NIH). The protocol for analysis was provided by The University of Queensland with the recommendations from the work[51]; **P* < 0.05 *vs.* corresponding control cells; #*P* < 0.05 *vs.* MCF7 cells.

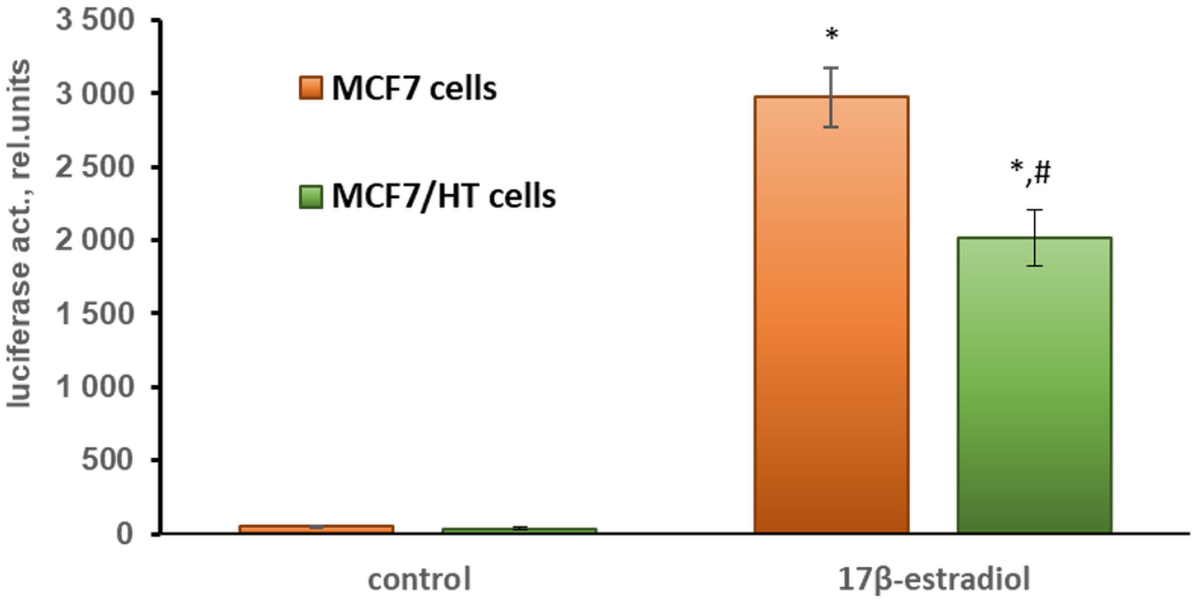

Figure 2. 17β-estradiol-induced activity of ERα in MCF7 cells assessed by gene reporter assay; **P* < 0.05 *vs.* control cells; #*P* < 0.05 *vs.* MCF7 cells treated with 10 nM 17β-estradiol. The mean values of three independent experiments are shown.

In subsequent experiments, we used MDA-MB-231 cells which are triple-negative; these cells are hormone-resistant *de novo*[53]. Analysis of βIII-tubulin (TUBB3) expression revealed the following trends: the level of TUBB3 expression was high and the same in all three studied cell lines, while the geometric mean of the marker expression differed dramatically [Figure 3].

Higher expression of TUBB3 was detected in triple-negative breast cancer MDA-MB-231 cells compared to hormone-responsive MCF7 cells [Figure 3]. The lowest TUBB3 expression was found in hormone-resistant MCF7/HT cells; the geometric mean of the marker was reduced by more than 2 and 4 times in comparison with MCF7 and MDA-MB-231 cells, respectively. Figure 3 shows one of the typical experiments for the evaluation of TUBB3 protein expression in the studied cell lines.

High TUBB3 expression strongly correlated ($P < 0.05$) with docetaxel resistance: IC_{50} value of docetaxel for MDA-MB-231 cells was greater than that for MCF7 cells, hormone-resistant MCF7/HT cells were the most sensitive to the drug [Figure 4].

In subsequent experiments, changes in signaling pathways induced by docetaxel were analyzed. MCF7 and MCF7/HT cells were treated with docetaxel and then protein expression was analyzed in the obtained samples. There are many ways of detecting apoptosis in cells, one of such approaches is to determine the level of cleaved PARP (poly (ADP-ribose) polymerase)[54]. The cleaved PARP may be considered a marker of apoptosis. As shown in Figure 5, incubation of cells with the drug leads to a dose-dependent increase in cleaved PARP expression. This indicates that docetaxel causes apoptosis. It is important to note that the accumulation of cleaved PARP is more pronounced in DCT-treated resistant cells (1.6-fold). Regulation of cell death pathways occurs with the participation of proteins from various families. The balance between proapoptotic and antiapoptotic proteins is usually observed in unchanged cells. Activation of antiapoptotic pathways is often detected in tumor cells. The expression of the antiapoptotic factor Bcl-2 was analyzed in MCF7 and MCF7/HT cells. Docetaxel caused a decrease in Bcl-2 expression in both cell lines, but the observed effects were more prominent in the resistant ones treated with 4 nM DCT (1.8-fold). Analysis of the expression of the proapoptotic protein Bad showed no differences between sensitive and resistant cells.

Docetaxel caused significant activation of ERKs in both cell lines ($P < 0.05$) and its effect was dose-dependent, as shown in Figure 5. The S6 kinase (S6K) is one of the effectors of the mammalian Target Of Rapamycin (mTOR); S6K regulates protein synthesis and cell growth. The overexpressed S6K was found in a variety of tumors and correlated to poor prognosis in cancers[55]. Amaral *et al.* described that S6Ks isoforms contribute to migration, viability, resistance to docetaxel and tumor formation of cancer cells[55]. We tested whether S6K is involved in the cell response to docetaxel. As shown in Figure 5, there were no differences in S6K expression between MCF7 and MCF7/HT cells. High activity of S6K was detected in all obtained samples, while docetaxel did not alter its activity or expression in both cell lines [Figure 5]. Intriguingly, the expression of the cell cycle regulator cyclin D1 decreased (2.8-fold) only in resistant cells after docetaxel treatment, while this marker was unchanged in parental MCF7 cells.

DISCUSSION

The interest in βIII-tubulin in cancer research is related to its role in drug tolerance of various tumours, particularly to taxanes, that block tubulin depolymerization, thereby increasing the content of polymerized tubulin and arresting cellular functions. Overexpression of βIII-tubulin was found in many tumours. TUBB3 expression was associated with a poor response to various drugs, including docetaxel, paclitaxel,

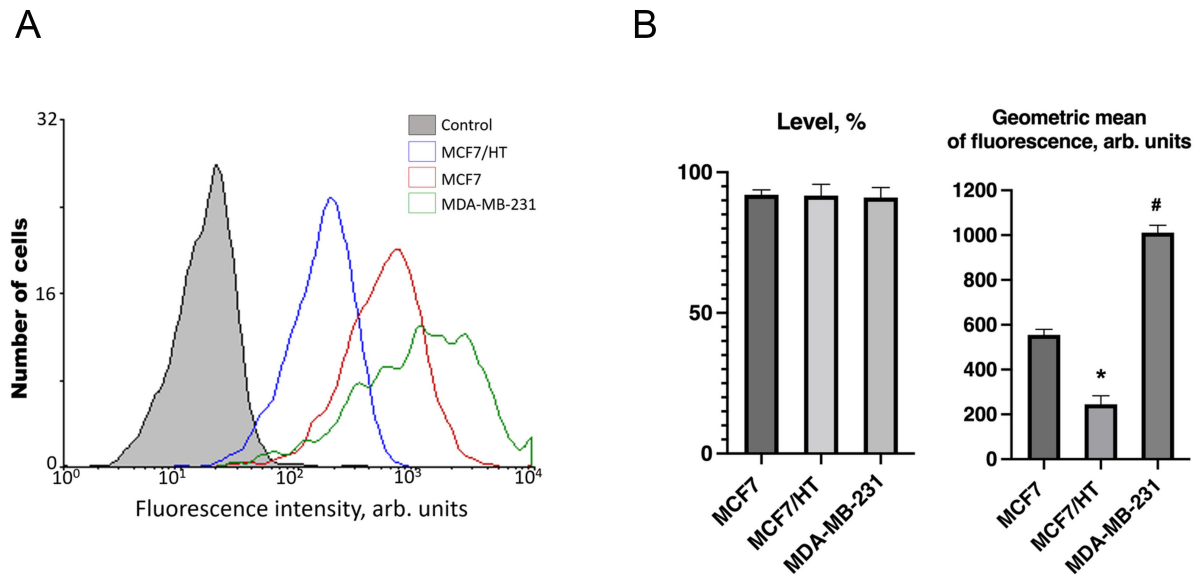

Figure 3. βIII-tubulin expression in breast cancer cell lines. (A) - Representative diagram of βIII-tubulin expression. The abscissa is the intensity of specific fluorescence in the channel of an FL6 flow cytometer (arb. units); the ordinate axis is the number of cells. Control - autofluorescence of samples; (B) - A difference in TUBB3 expression was revealed between the cell lines, and the intensity of the marker expression increased in the following order (n = 3): MCF7/HT < MCF7 < MDA-MB-231 (approximately, 1:2:4). *P < 0.05 *vs.* MCF7 and MDA-MB-231, #P < 0.05 *vs.* MCF7 and MCF7/HT.

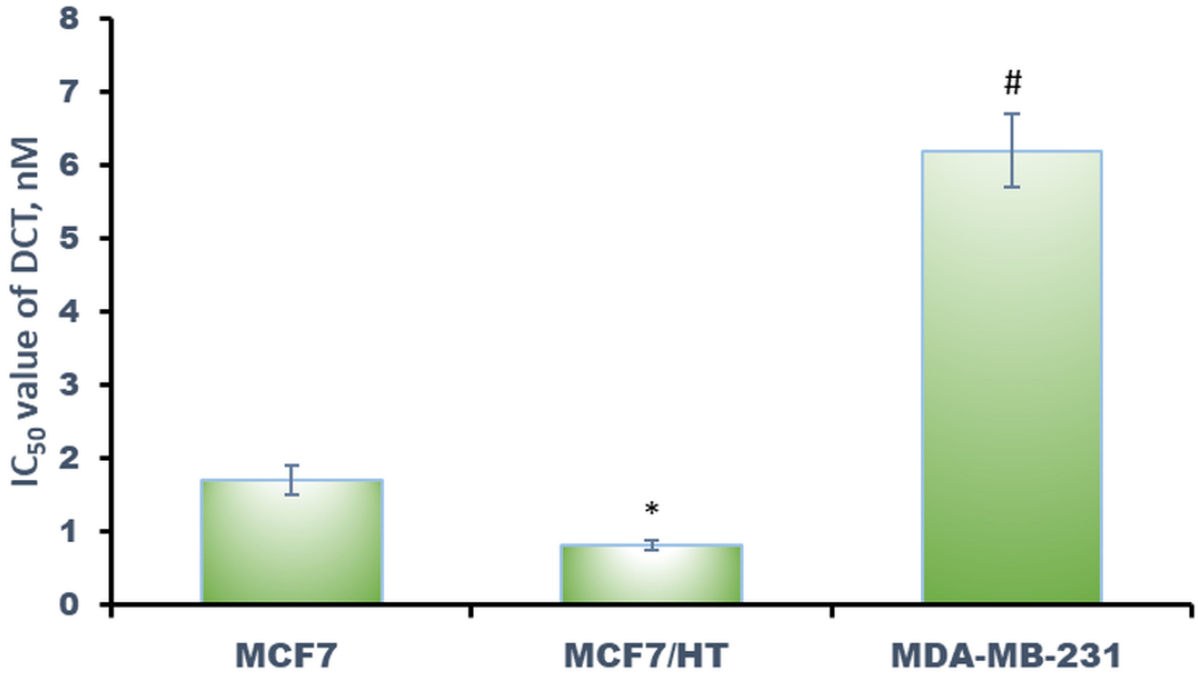

Figure 4. Effects of docetaxel (DCT) against breast cancer cells. *P < 0.05 *vs.* MCF7 and MDA-MB-231, #P < 0.05 *vs.* MCF7 and MCF7/HT. The results for three independent experiments are shown.

vinca alkaloids, cisplatin, etoposide, and doxorubicin. βIII-tubulin overexpression was associated with a poor response to microtubule-targeting agents and a shorter overall and progression-free survival in various

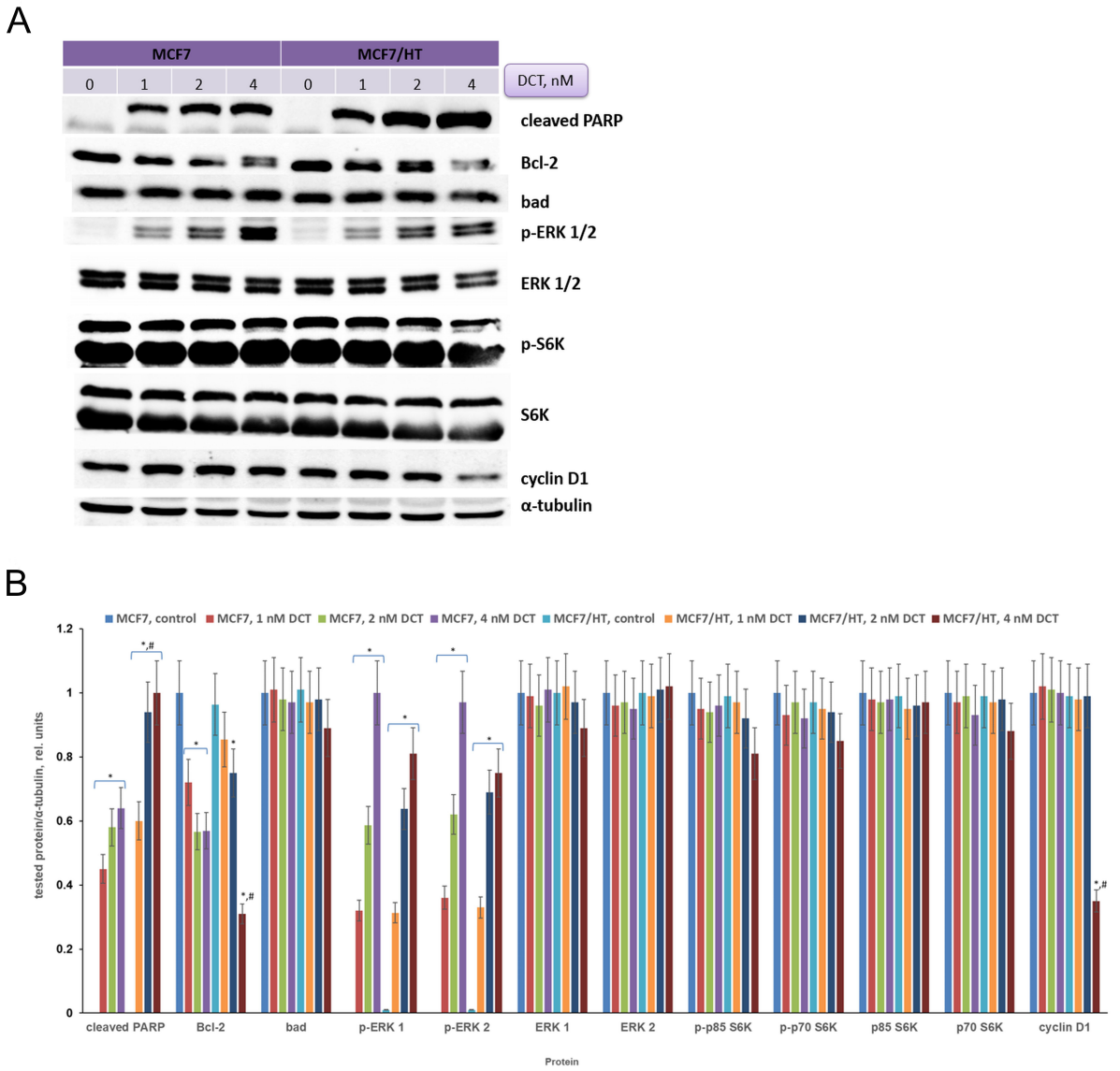

Figure 5. Effects of docetaxel on signaling in MCF7 and MCF7/HT cells. (A) - Representative immunoblotting images of MCF7 and MCF/HT cells with antibodies against cleaved PARP, Bcl-2, Bad, p-ERK 1/2, ERK 1/2, p-S6K, S6K, and cyclin D1; the cells were treated with docetaxel (DCT) for 24 h and then subjected to immunoblot analysis. Antibodies against α-tubulin were used as loading control. (B) - Densitometry for immunoblotting data (n = 3); $^{*}P < 0.05$ *vs.* corresponding control cells; $^{\#}P < 0.05$ *vs.* corresponding DCT-treated MCF7 cells.

cancers, including bladder, lung, ovarian, breast, prostate, and rectal cancers. Moreover, βIII-tubulin expression was positively correlated with lymphatic metastasis and tumor differentiation[23]. TUBB3 is also involved in docetaxel (DCT) and cabazitaxel (CBZ) resistance. LY294002, a PI3K inhibitor, re-sensitized DCT-resistant cell lines to docetaxel and CBZ-resistant cell lines to cabazitaxel (CBZ). The combination of DCT/CBZ and LY294002 could be potential strategy for the treatment of prostate cancer[56].

Several approaches have been developed to enhance the action of docetaxel. In preclinical projects, researchers use specific nucleotides against signaling RNAs to modulate the action of docetaxel. Dr Razi Soofiyani *et al.* described very interesting experiments with siRNA-mediated silencing of CIP2A[57,58]. CIP2A silencing enhanced the sensitivity of cancer cells to DCT by strengthening drug-induced cell growth

inhibition and apoptosis. The authors concluded that CIP2A silencing may potentiate the antiproliferative effects of docetaxel and this might be a promising therapeutic approach in prostate cancer treatment. cMET is considered as a target in the work by Dr Majidi Zolbanin *et al.*[59]. The mucin1 aptamer-conjugated chitosan nanoparticles, containing docetaxel and cMET siRNA, were suggested by authors for the treatment of mucin1-positive metastatic breast cancers.

The molecular pathway of ERKs plays a crucial role in the growth and death of normal and cancer cells[60]. Depending on the stimulus, activity of ERKs mediates various antiproliferative signals, such as apoptosis and autophagy[61-63]. Chemotherapeutic and other DNA damage agents, including etoposide, doxorubicin, ionizing radiation and ultraviolet irradiation (UV), activate protein kinase ERK1/2 in various cells[61,64]. Previously published data are in good agreement with our observations on ERK activity in cells after DCT treatment. Lucie Chauvin *et al.* discuss the possible role of ERK in maintaining the survival of docetaxel-treated cells in the work[65]. Authors demonstrated that docetaxel supports a survival signaling pathway through a mechanism depending on PKC and ERKs in the MDA-MB-231 breast cancer cells. Thus, the use of combinations of docetaxel with ERK inhibitors could be a promising strategy for future studies of breast cancers.

The relationship between βIII-tubulin expression and taxane-resistance of tumours is being extensively studied. For example, Maahs *et al.* have studied βIII-tubulin expression as a predictor of resistance in patients with metastatic castration-resistant prostate cancer and have revealed that patients with a high expression of TUBB3 had a lower survivability and worse response rates to docetaxel as indicated by a 10% or greater decrease in prostate-specific antigen (PSA) compared to a 50% or more decrease in patients with a low βIII-tubulin who have a better response rate to docetaxel[66].

De Donato *et al.* have considered TUBB3 as a gateway for survival PIM1 signals[67]. The cells are exposed to microenvironmental stressors and PIM1 was incorporated into the cytoskeleton through GBP1 and βIII-tubulin, which ultimately leads to drug resistance. Moreover, De Donato *et al.* have found a statistically significant up-regulation of class III beta-tubulin in the paclitaxel-resistant ovarian tumors[67]. Similarly, Roque *et al.* have found that TUBB3 overexpression in clear cell carcinoma of the ovary discriminates poor prognosis. High TUBB3 expression is a marker for sensitivity to patupilone and may contribute to resistance to paclitaxel[68].

The results of our study are consistent with the evidence described above: the cells with the highest βIII-tubulin expression (MDA-MB-231) were resistant to docetaxel and the cells with the lowest βIII-tubulin expression (MCF7/HT) were sensitive to docetaxel. Several researches show that βIII-tubulin expression in cancer cells is regulated by hormones. Saussede-Aim *et al.* showed that 17β-estradiol exposure causes an up-regulation of βIII-tubulin in ERα-positive MCF7 breast cancer cells, and estrogen receptor modulators (e.g. tamoxifen) reduce the βIII-tubulin level in ERα-positive breast cancer cells, but did not affect the βIII-tubulin level in ERα-negative MDA-MB-231 cells[69]. This mentioned observation is in good agreement with our data. We have shown that estrogen receptor α activity is decreased in the hormone-resistant cells, and the hormone dependence of the cells decreases accordingly. A decrease in βIII-tubulin expression may be associated with these changes in hormone signaling of cancer cells. There is also evidence that androgens modulate TUBB3 expression: in prostate cancer cells and patient tumors, androgen ablation correlates with high TUBB3 levels[70]. In another work, an increase in βIII-tubulin was revealed in androgen-starved and androgen receptors knockdown human prostate adenocarcinoma cells LNCaP[71].

Consistent with these facts, MCF7/HT in our study had the lowest activity of ERα along with the lowest β III-tubulin level; on the contrary, MDA-MB-231 with no ERα at all had the highest βIII-tubulin level. Docetaxel affected the expression of a number of signaling proteins in MCF7 and MCF7/HT breast cancer cells. The accumulation of cleaved PARP (a marker of apoptosis) and Bcl-2 downregulation were more pronounced in resistant cells. Moreover, the expression of cyclin D1 decreased only in resistant cells after docetaxel treatment, while this marker was unchanged in parental MCF7 cells. Interestingly, according to several clinical trials, a high level of TUBB3 is associated with negativity for estrogen and progesterone receptors in breast cancer patients[72,73] and, as a result, with worse disease-free and overall survival[73,74].

In conclusion, high Akt activity (a 2.2-fold increase) and decreased activity of the estrogen receptor α (1.5-fold) were found in established hormone-resistant MCF7/HT cells. It is intriguing that the expression of TUBB3, metastasis-associated tubulin, was lowered in the hormone-resistant cells. The hormone-resistant cells were characterized by high sensitivity to tubulin polymerization inhibitor docetaxel, belonging to taxanes. The significant accumulation of cleaved PARP (1.6-fold) and Bcl-2 downregulation (1.8-fold) were revealed in DCT-treated MCF7/HT cells. Thus, further development of taxane-based chemotherapy for hormone-resistant cancer with low TUBB3 expression looks highly promising.

DECLARATIONS

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Authors' contributions

Contributed reagents/materials/analysis tools and critically revised the manuscript: Bogush TA, Krasil'nikov MA, Mikhaevich EI

Performed the experiments, analyzed all the data and amended the revised version: Scherbakov AM, Basharina AA, Sorokin DV, Mikhaevich EI, Mizaeva IE, Mikhaylova AL

Conceived and designed the experiments: Scherbakov AM

Read and approved the final manuscript: Scherbakov AM, Basharina AA, Sorokin DV, Mikhaevich EI, Mizaeva IE, Mikhaylova AL, Bogush TA, Krasil'nikov MA

Availability of data and materials

The material used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Mechanisms involved in cancer stem cell resistance in head and neck squamous cell carcinoma

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Abstract

Despite scientific advances in the Oncology field, cancer remains a leading cause of death worldwide. Molecular and cellular heterogeneity of head and neck squamous cell carcinoma (HNSCC) is a significant contributor to the unpredictability of the clinical response and failure in cancer treatment. Cancer stem cells (CSCs) are recognized as a subpopulation of tumor cells that can drive and maintain tumorigenesis and metastasis, leading to poor prognosis in different types of cancer. CSCs exhibit a high level of plasticity, quickly adapting to the tumor microenvironment changes, and are intrinsically resistant to current chemo and radiotherapies. The mechanisms of CSC-mediated therapy resistance are not fully understood. However, they include different strategies used by CSCs to overcome challenges imposed by treatment, such as activation of DNA repair system, anti-apoptotic mechanisms, acquisition of quiescent state and Epithelial-mesenchymal transition, increased drug efflux capacity, hypoxic environment, protection by the CSC niche, overexpression of stemness related genes, and immune surveillance. Complete elimination of CSCs seems to be the main target for achieving tumor control and improving overall survival for cancer patients. This review will focus on the multi-factorial mechanisms by which CSCs are resistant to radiotherapy and chemotherapy in HNSCC, supporting the use of possible strategies to overcome therapy failure.

Keywords: Head and neck squamous cell carcinoma, cancer stem cell, chemotherapy, radiotherapy, therapy resistance

INTRODUCTION

Head and neck malignancies are now the seventh most common type of cancer worldwide[1]. More than 90% of head and neck tumors are derived from mucosa epithelium and are diagnosed as squamous cell carcinoma (HNSCC). Although sharing identical histological subtypes, HNSCC can be divided into at least two genetic subclasses based on the absence or participation of human papillomavirus (HPV) in carcinogenesis[2]. The oral cavity represents the main subsite for HPV-negative tumors and the oropharynx for HPV-positive ones[3]. Moreover, these subgroups also differ in clinical profile, tumor behavior, survival rates, and prognoses[4].

The mainstay treatment for HNSCC consists of surgery with adjuvant or neoadjuvant chemotherapy and radiotherapy. More recently, immunotherapy with checkpoint inhibitors has been indicated for recurrent and metastatic HNSCC with promising results, although only a subset of patients with HNSCC has shown a response to this therapy[5]. TNM stage of the disease and anatomic subsites influence therapeutic options for HNSCC. While radical surgeries are the first choice for locally advanced oral cancer, the main treatment for oropharyngeal tumors is chemoradiotherapy, regardless of HPV status. Nowadays, transoral surgeries (robotics and laser microsurgery) have also been performed in the oropharynx region[6].

Despite the advances in current therapy, the prognosis of HNSCC remains poor. More than half of patients die from the disease or complications within a short period, varying from a few months to five years[7]. The primary cause of mortality is related to resistance to therapy which leads to local recurrence, cervical lymph node metastasis, and occasionally, distant organ metastasis[6]. Tumor heterogeneity and cancer stem cells (CSCs) are known to enhance metastatic dissemination and therapeutic resistance, contributing to lethality[8].

CSCs represent a small but critical subpopulation of cells in the tumor capable of self-renewal and multilineage differentiation and regenerating a tumor when serially transplanted into mice models[9]. Since tumors can regrow from a single CSC, cancer treatment success may be attributed to the complete eradication of CSCs populations[8].

Besides, CSCs also demonstrate cellular plasticity; they can reversibly switch between different stem cell phenotypes and between a stem and non-stem cell state[10]. CSCs activity is modulated by different signals and cellular interactions provided by the tumor microenvironment, allowing CSCs to achieve highly invasive and aggressive behavior or resist conventional therapies. Thus, activating the Epithelial-to-Mesenchymal Transition (EMT) program by the CSCs represents a valuable strategy to promote invasion, metastasis, and treatment resistance[10-12].

CSCs may originate from adult stem cells or progenitor cells in which the accumulation of mutations over time leads to the activation of transcriptional gene signatures and signaling pathways related to the maintenance of stem cell phenotype and malignant transformation[13-15]. Moreover, differentiated cells can also acquire stemness traits due to genetic instability throughout their division process and dedifferentiate, acquiring stem cell properties[16,17]. It is essential to highlight that malignant cells can dedifferentiate and acquire stem cell characteristics under challenging situations, including exposure to chemotherapy and radiotherapy[18].

In HNSCC, Prince *et al.* first described the presence of a small fraction of CD44-positive cells capable of generating new tumors when inoculated in immunocompromised mice and re-establishing original tumor heterogeneity[19]. Moreover, this subpopulation expressed the *Bmi1* gene, a stemness marker involved in tumorigenesis and self-renewal[19]. Since then, other common HNSCC CSC markers, such as CD44, ALDH1, CD133, c-Met, and Bmi-1, have been described[20-22]. ALDH1 is considered a highly specific CSC marker, mainly when evaluated with CD44[20]. Moreover, based on CD44 and EpCAM expression levels, CSCs in oral squamous cell carcinoma (OSCC) seem to switch between two distinct phenotypes. First, $CD44^{high}/EpCAM^{high}$ presents an epithelial morphology and colony formation capability, and second, $CD44^{high}/EpCAM^{low}$ has a mesenchymal morphology (EMT profile) with high invasive potential, metastasis and radioresistance ability[10,23]. More recently, LIN28A and LIN28B proteins, located in the cytoplasm and nucleus/nucleoli, respectively, were identified as reprogramming factors that can lead to the de-differentiation of malignant oral squamous cancer cells into CSCs and contribute to their immune evasion[24].

For other types of cancers, distinct CSCs can be identified and isolated by fluorescence-activated cell sorting (FACS) using phenotypic surface markers alone or in combination. More than 40 surface markers are known to identify CSCs in solid tumors, and the majority are derived from embryonic or adult stem cells[25]. In general, high positivity of CD44, CD24, CD133, CD90, EpCAM, and Aldehyde Dehydrogenase 1 (ALDH1), and elimination of Hoechst 3334 dye *via* ABC transporters are the most used markers[26]. The isolated CSCs can be propagated *in vitro* as spheroids or used in organoid cultures. Moreover, spheroid cultures are CSCs enriched, show self-renewal ability *in vitro* and *in vivo,* and generate tumors that resemble the original tumor heterogeneity and differentiation[27].

More recently, in addition to the conventional 2D cell culture, 3D culture models have been used to represent tumor microenvironment heterogeneities properly and reproduce patients' tumor behavior. Engelmann L. *et al.* developed a 3D Organotypic Co-Culture (3D-OTCs) utilizing HNSCC fresh tissue (non-HPV driven and HPV-driven) placed on top of dermal equivalents (human fibroblasts cultured on a viscose fiber fabric) and analyzed samples' behavior[28]. All non-HPV-driven 3D-OTCs were capable of proliferating cancer cells for up to 21 days and exhibited a heterogeneous, invasive, and expansive growth pattern[28]. In the same context, Miserocchi G. *et al.* developed a 3D culture using HPV-positive and HPV-negative HNSCC cells in a collagen-based scaffold. They suggested that the 3D model might induce more mesenchymal phenotypes than 2D cultures[29]. Also, in this study, HPV-negative cells presented an upregulation of FLT1 and ABCA3 when seeded in scaffolds, overexpressed EMT-related genes, and increased migration ability compared to HPV-positive cells[29]. Based on these findings, collagen-based scaffolds seem to activate drug-resistance mechanisms reassuring the ability of 3D scaffolds to reproduce HNSCC tumor microenvironment impeded by other *in vitro* systems. Accordingly, regarding response to treatment analyses, 3D culture is promising in the future of HNSCC and CSC research.

Several associations between clinicopathological characteristics and CSCs have been appointed in HNSCC, including tumor size, regional and distant metastases, perineural invasion, radiation failure, and poor disease-free survival[30]. A previous study of our group explored CSCs markers in tongue tumors and found that the overexpression of CD44 was related to worst overall survival, and Nanog and Oct4 were associated with regional metastasis and death[31]. Ma *et al.* suggested that $CD133^{+}$ cells could be responsible for aggressiveness and chemoresistance in oral tumors[32]. A meta-analysis study by Fan *et al.* showed that the CSCs markers, CD133, Nanog, and Oct4, could have a prognosis value in HNSCC patients[33]. In light of recent events in CSCs markers, there is now some discovery about non-coding RNAs (ncRNAs) used as biomarkers of cancer development and tumor stage determination[34].

MicroRNAs are a type of sncRNA that regulate biological processes. Each miRNA can control target genes and accentuate their potential influence on almost every genetic pathway. Hsieh PL *et al.* demonstrated that ncRNA molecules associated with CSCs are responsible for acquiring and maintaining cancer stemness[35]. Let-7 genes family act as a tumor suppressor. Lin28B-let-7 pathway positively regulates the expression of stemness factors Oct4 and Sox2; it causes a switch of non-CSCs to CSCs with tumor starting and self-renewal characteristics in oral CSC[36].

MicroRNA-200 family is another group of genes related to CSC; expression levels of miR-200c were downregulated in ALDH1+/CD44+ HNSCC with BMI1 overexpression. Also, an expression of let-7c or let-7d in oral CSCs suppressed stemness and the radio/chemoresistance hallmarks through suppression of IL-8 or EMT markers, respectively[37,38]. MicroRNA-494 acts as a tumor suppressor or oncogenic factor. An increase of miR-494 can inhibit ALDH1 activity, CD133 positivity, and other stemness signatures in ALDH1+CD44+ oral cancer cells. In the same way, activation of miR-494 inactivates Bmi-1 and ADAM10 expression in OSCC-CSCs[39]; also, miR-494-3p may enhance the radiosensitivity and induce a senescence pathway in oral cancer cells[40].

In this scenario, it is essential to highlight that CSCs are not easily eliminated by conventional therapies, meaning that after the effective depletion of the bulk of the tumor, residual CSCs populations may survive, drive and sustain cancer recurrence, invasiveness, and therapy resistance[41]. Moreover, CSCs are considered intrinsically resistant to chemo and radiotherapy. It is also possible that the CSCs and their close descendants give rise to therapeutic-resistant malignant cells that accumulated mutations caused by genotoxic therapies[42]. CSCs adopt different strategies to overcome the challenges imposed by treatment, including the acquisition of dormancy, which is influenced by the CSC niche and immune surveillance, increased drug efflux capacity, activation of DNA repair machinery and decreased activation of apoptosis[43]. This review will focus on the mechanisms that lead to CSC resistance to radiotherapy and chemotherapy in HNSCC.

RADIORESISTANCE AND CSC

In HNSCC patients, radiotherapy (RDT) is a common choice of treatment to achieve cancer control after surgery and/or current chemotherapy[6]. Usually, on weekdays patients receive a dose of 70 Gy that can be administered through standard fractionation (2 Gy, once a day) or via accelerated fractionation and hyperfractionation (twice a day)[44]. Fractionation guarantees that cancer cells will eventually be exposed to radiation in all cell cycle phases, favoring DNA damage and cell fate. Nevertheless, this process also activates important protein regulators of DNA damage response, such as ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related protein (ATR), which will be decisive in treatment response[45].

Tumor response or failure to ionizing radiation is mainly associated with the classical 4 R's of radiobiology: repair of sublethal DNA damage, reassortment of cells in the cell cycle, cell repopulation, and reoxygenation of hypoxic areas[46]. Efficient cell death by RDT depends on producing unrepairable damage involving DNA double-strand breaks (DSBs); however, most radiation-induced DNA damage is sublethal. DNA repair systems include base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), non-homologous end joining (NHEJ), and mismatch repair (MMR) pathways[47]. In this context, CSCs seem to hold elevated levels of proteins responsible for NHEJ and HR and an increased DSB repair capacity[23].

If tumor recurrences occur within six months following radiation, tumors are considered radioresistant[48]. Mechanisms involved in radioresistance are not fully understood, but accumulated evidence indicates that

cancer stem cells (CSCs) are decisive in this process[46,49]. In general, therapeutic resistance refers to the ability of cancer cells to recover and repair DNA damage and regrow after tumor therapy[50], being higher in CSCs than non-CSC[51]. This ability is mainly related to the increased regulation of DNA repair genes, DNA-damage checkpoints, and anti-apoptotic proteins[52,53].

Furthermore, it has been recognized that a CSC subpopulation exhibiting a mesenchymal profile ($CD44^{high}/CD24^{low}$) presents an even higher level of DNA repair following RDT[23]. Besides, irradiation activates stemness pathways and induces CSC phenotypes in non-stem cancer cells. Up-regulation of CSCs genes, such as Sox2 and Oct3/4, may be observed after radiation, contributing to tumor radioresistance[53]. The plasticity of CSCs dramatically interferes with identifying and eliminating CSCs during cancer therapies[54].

Radiation promotes an arrest of CSCs in the G2/M phase, which allows active DNA repair. Moreover, after radiation, there is a noticeable discrepancy between the higher rates of self-renewal and proliferative abilities of CSCs compared to their lower apoptosis activation, favoring tumor growth[52]. In oral cancer cell lines, changes in CSCs content (ALDH+) are associated with an increase in the rates of sub-lethal damage repair (SLDR), which enables efficient cell repair and reduces tumor control capabilities[55]. Duration of the exposure to the fractionated dose-delivery of radiation seems to influence radioresistance mechanisms driven by SLDR, suggesting that reduced overall dose-delivery time on radiotherapy could favor CSCs control[55].

Besides the DNA repair process, activation of checkpoint responses after radiation damage also participates in the radioresistance of several tumors, including HNSCC. Cell cycle progression is delayed to allow DNA repair through activation of signaling pathways such as ataxia telangiectasia mutated (ATM)-checkpoint kinase 2 (Chk2) and ATM-Rad3-related (ATR)- checkpoint kinase (Chk1)[56]. CSCs appear to enhance response to DNA damage activating Chk2 in invasive oral cancer[23]. Inhibition of Chk1 was suggested as a therapeutic target in HNSCC that contributes to the failure of DNA replication and intensification of DNA damage[57].

Induction of apoptosis represents one of the primary mechanisms by which cancer cells are eliminated in cancer therapies[58]. Reduced cleaved caspase proteins showed the apoptotic resistance of CSCs in oral cancer after irradiation[23]. Resistance mechanisms evolving upregulation of anti-apoptotic proteins such as Bcl-2 and inhibitor of apoptosis (IAP) are commonly found in tumor cells, especially in CSCs[59]. Radiation can activate X-linked IAP (XIAP), another IAP family member that inhibits apoptosis mediated by mitochondrial and caspase-3 pathways[60]. Besides apoptosis regulation, Bcl-2 family members also participate in cell migration, invasion, and metastasis[61]. In this focus, an inhibitor of Bcl-2 combined with Cetuximab and radiation showed excellent results in eliminating CSCs in HNSCC cell lines[62].

Another widely studied mechanism of CSCs contributing to radioresistance and poor prognosis in HNSCC is related to hypoxia, i.e., low oxygen levels caused by insufficient blood supply to tumor tissues[63,64]. A hypoxic tumor environment can interfere directly with the potential of radiation to damage DNA cells and indirectly regulate the expression of genes related to aggressiveness and response to treatment. Additionally, hypoxia is essential in protecting the CSCs niche from radiation effects and in acquiring and maintaining CSC-like phenotype[65].

In HNSCC, hypoxia-inducible factor-1a (HIF-1α), a transcriptional regulator of oxygen homeostasis, is enhanced in CSCs subpopulations in response to radiation[66]. Furthermore, hypoxia upregulates CSCs

genes such as *Sox2* and *Nanog*, consequently contributing to the survival of tumor cells after radiation[67]. Linge *et al.* showed a correlation between high tumor recurrence after postoperative radiochemotherapy in locally advanced HNSCC patients, increased expression of CSCs markers, and high hypoxia-induced gene signature expression[68]. Strategies for hypoxic modifications such as hyperbaric oxygenation or nitroimidazoles significantly reduced locoregional recurrence after radiation in HNSCC[69].

In the same context, reactive oxygen species (ROS) and redox-regulatory mechanisms can regulate DNA damage and resistance to irradiation. Accumulation of ROS and DNA damage of cancer cells is associated with the effectiveness of radiotherapy[70]. Unlike non-CSCs, CSCs present a high antioxidant capacity that coordinates the activity of free-radical scavengers and protects cells from induced-radiation death[70,71]. This low ROS state presented by CSCs is also related to the quiescent state of HNSCC stem cells and enhanced tumorigenic potentials *in vitro* and *in vivo*[72]. Interestingly, GDF15 (growth differentiation factor 15), a member of the TGF-β superfamily, participates in ROS suppression in HNSCC, contributing to radioresistance and acquisition of the CSC phenotype[73]. Boivin *et al.* showed that redox-modulating by inhibiting GSH antioxidant system previous to radiation is an accurate strategy to eliminate highly tumourigenic CSCs[74].

Considering the better prognosis of HNSCC HPV-positive patients, it seems that HPV may influence several molecular mechanisms involved in CSC's radiosensitivity[75,76]. Rieckmann *et al.* demonstrated a limited capacity of DSB repair in HPV/p16-positive cancer cells[77]. HPV-positive tumors are believed to present less radioresistant CSCs subpopulations due to their reduced repopulation ability during radiation therapy[78]. Reid *et al.* explored irradiation behavioral responses of CSCs with $CD44^+$ $ALDH^+$ phenotype in 6 HPV positive and negative HNSCC cell lines[79]. Their principal findings showed that HPV status did not influence the inherent proportions of CSCs, which were changed in both groups in response to radiation. HPV-negative samples showed a significant increase in CSCs densities, probably reflecting their remarkable repopulating ability after treatment[79]. Other studies demonstrated that HPV-negative cell lines seem more capable of dedifferentiating from non-CSCs to CSCs in response to radiation than HPV-positive cell lines[80]. In addition, low levels of functional TP53 expressed by HPV-positive cells may contribute to inducing apoptosis following radiotherapy[81].

In an attempt to address this issue, the literature has found that cisplatin-sensitization has helped overcome resistance to radiation in many patients. In a recent study, Routila *et al.* appointed *Oct4* as a good marker for identifying radioresistance and cisplatin-sensitive tumors, which could help distinguish patients who should receive cisplatin-sensitization from those who would not benefit from this therapy[82]. In summary, Oct4 positivity reduced cancer cell apoptosis, favoring cell viability after irradiation. At the same time, Oct4 can contribute to cisplatin mechanisms inhibiting DNA repair activation[82]. In radioresistance, Oct4 driving activates the oncogene Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A), which promotes malignant cell growth and proliferation[50].

Despite technological developments, RDT still promotes long-term toxicities compromising the quality of life and is often associated with potential tumor resistance[6,83]. CSCs act as key players in regulating different mechanisms of DNA damage repair and other regulators of cell death after irradiation, such as hypoxia, apoptosis, and ROS [Figure 1]. At this point, we believe that RDT is insufficient to eliminate CSCs in HNSCC, explaining the high recurrence rates of these tumors. Thus, further investigation is required to comprehend and overcome CSC's radioresistance and improve treatment success and overall survival in cancer patients.

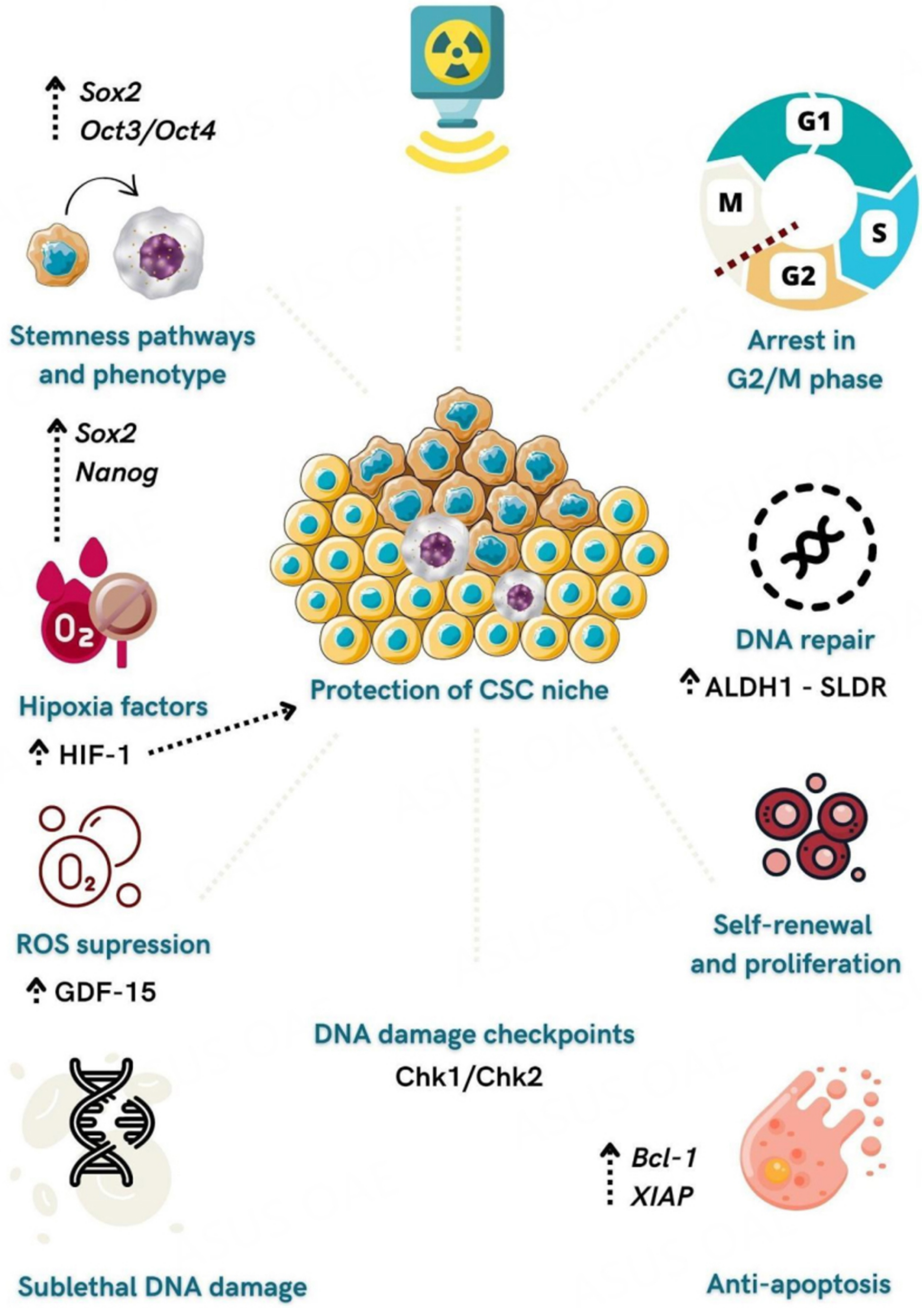

Figure 1. Mechanisms related to CSCs radioresistance in HNSCC. Radiation can activate stemness pathways such as Sox-2 and Oct3/4 and induce CSC phenotype in non-stem cancer cells. Radiation promotes an arrest of CSCs in the G2/M phase and activates Chk2 and Chk1, which delays cell cycle progression and allows DNA repair. Overexpression of CSC marker ALDH1 leads to increased rates of sub-lethal damage repair (SLDR), enabling efficient cell repair and reducing tumor control capabilities. CSCs upregulate anti-apoptotic proteins such as Bcl-2 and X-linked inhibitors of apoptosis (XIAP). Hypoxia upregulates CSCs genes (*Sox2* and *Nanog*) and is essential in protecting the CSCs niche from radiation effects. GDF15 (growth differentiation factor 15) participates in ROS suppression in HNSCC, contributing to radioresistance and acquisition of the CSC phenotype.

CHEMOTHERAPY RESISTANCE AND CSC

HNSCC in stage I or II (early tumors) is curable with higher survival rates after surgery or radiotherapy alone. In contrast, over 60% of stage III or IV HNSCC (locoregionally advanced) require advanced

therapeutic options such as surgery followed by radiotherapy with or without chemotherapy[7]. Currently, the standard chemotherapy regimens for stage III or IV, as well as recurrent and metastatic HNSCC, are based on cisplatin, 5-fluorouracil (5-FU), and docetaxel/paclitaxel[84-86].

Chemotherapeutic drugs exert different biological effects on tumor cells, relying on specific mechanisms of action. Cisplatin is a platinum-based alkylating agent that creates inter- or intra-strand cross-links or transfers alkyl groups to the guanine residues of DNA, generating mispairing formation in DNA bases and avoiding strand separation during DNA synthesis[87]. On the other hand, 5-FU is a pyrimidine antagonist antimetabolite that interferes with essential biosynthetic pathways, disturbs the DNA/RNA synthesis, or causes the formation of DNA strand breaks through inhibition of particular enzymes or incorporation of false structural analogs of pyrimidine/purine into DNA[88]. Docetaxel is a topoisomerase II inhibitor that impairs DNA replication and causes DNA strand breaks. Paclitaxel is a taxane that modifies the function/formation of spindle microtubules by inhibition of nuclear division (mitotic arrest in metaphase), leading to cell death[87]. In this context, it is essential to highlight that most chemotherapeutics' success relies on the drugs' ability to decrease tumor size or induce short-term remission. This measure of success is intuitive, and many medications evaluated by these criteria are used in effective chemotherapeutic regimens[89].

Although the chemotherapeutic scenario seems broad, mortality from HNSCC continues to rise worldwide[90]. As reviewed by Bukowski *et al.*, part of this problem may be a reflection of drug resistance, which leads to a reduction of the therapeutic efficacy and is related to over 90% mortality of cancer patients[91]. Multi-drug resistance (MDR) of cancer cells during chemotherapy can be associated with a variety of mechanisms, including enhanced efflux of drugs, drug activation or inactivation, genetic factors (gene mutations, amplifications, and epigenetic alterations), growth factors, increased DNA repair capacity, inactivation of apoptosis machinery, increased autophagy, and elevated metabolism of xenobiotics, or even any combination of these mechanisms[91-93]. In addition, establishing a tumor microenvironment (TME) promotes tumor progression and chemoresistance through a collection of soluble proteins and insoluble vesicles secreted by tumor cells. This cell-to-cell communication among various cell types required to form the TME, such as mesenchymal stromal cells, immune cells, and vascular endothelial cells, influences the function of cells in the TME, shapes the premetastatic niche, and is an essential contributor to the development of chemoresistance[94].

Tumor heterogeneity is a significant complicating factor in cancer treatment and is also strictly associated with chemotherapy resistance, impacting poor prognosis for HNSCC patients[95]. Specifically, the presence of the CSCs has been associated with resistance to chemotherapeutic agents such as cisplatin, bortezomib, etoposide, 5-FU, and doxorubicin[92,96]. Most importantly, many studies have demonstrated that treatment with these drugs enhances the CSCs fraction in different solid tumors and favors EMT traits, leading to treatment resistance and cancer progression[97,98]. In addition, the acquisition of resistance to a specific drug generally tends to multiply resistance to unrelated compounds in CSCs and malignant cells, which under treatment pressure, can acquire a stem-like phenotype and become therapeutic resistant[18].

CSCs were identified as crucial players in the acquisition of drug resistance and unresponsiveness to current chemotherapies against cancer by activating different cellular signaling pathways and mechanisms [Figure 2]. The main reasons found in the literature rely on intrinsic properties of CSCs, such as the (1) inherent quiescent state that enables them to evade the actions of drugs that target rapidly proliferating cells; (2) high levels of drug efflux pumps and detoxifying enzymes; (3) increased DNA self-repair capacity; (4) specific expression of anti-apoptotic and prosurvival proteins; (5) acquisition of the EMT-phenotype; (6)

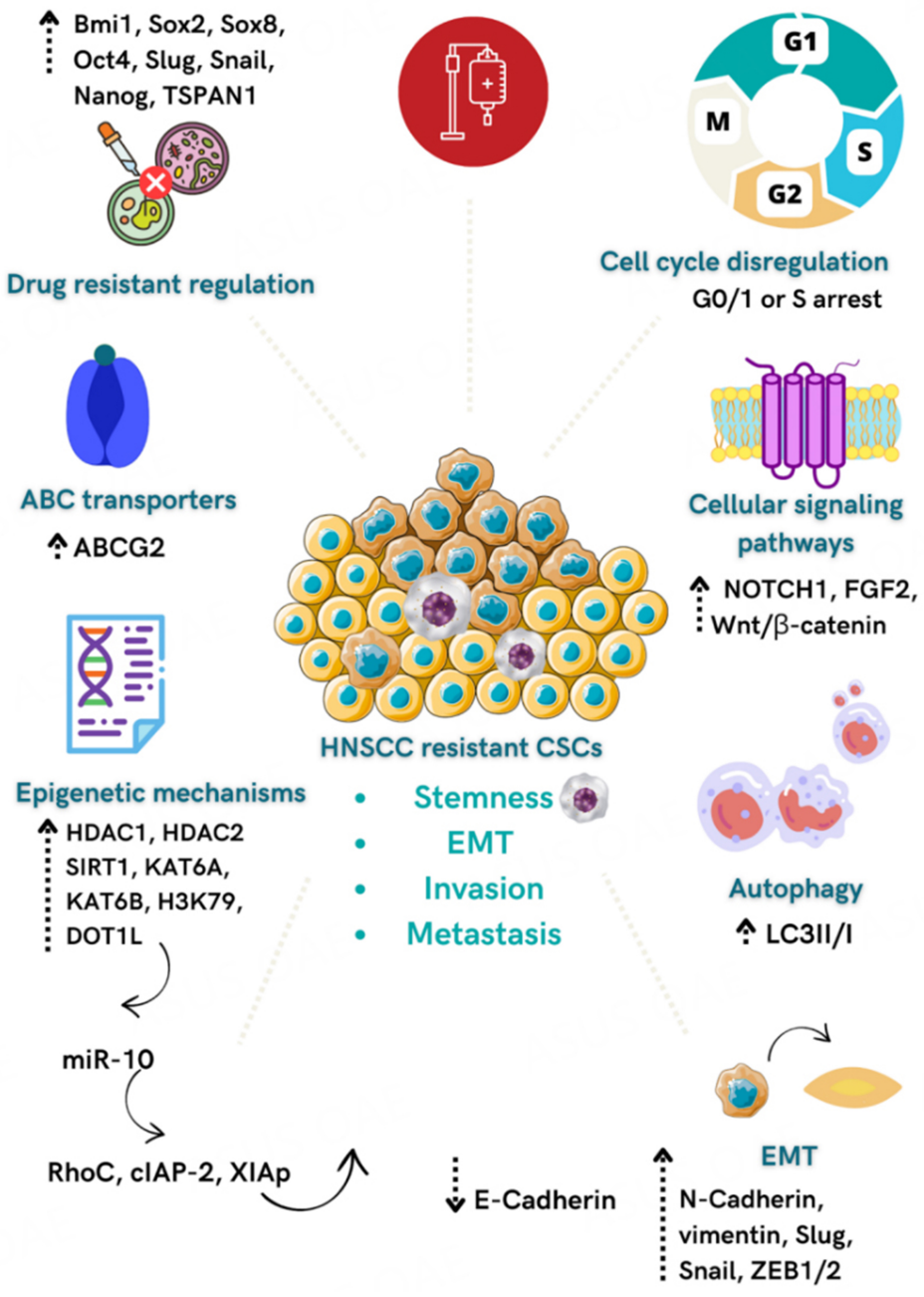

Figure 2. Mechanisms related to CSCs' chemoresistance in HNSCC. Overexpression of *Bmi1*, *Sox2*, *Sox8*, *Oct4*, *Slug*, *Snail*, *Nanog*, and *TSPAN1* genes leads to the acquisition of drug resistance and stemness, EMT, and metastasis. CSCs activate signaling pathways such as the NOTCH1, FGF2, and Wnt/β-catenin to promote chemoresistance and stemness. Increased expression of ABC transporters, mainly ABCG2, the activation of EMT, cell cycle deregulation, increased autophagy, and activation of epigenetic mechanisms, such as up-regulation of miR-10, are involved with CSC's chemoresistance in HNSCC.

oxidative modulation; (7) epigenetic modifications and (6) activation of the specific signaling pathways[90,99-103]. In addition, the role of the TME in sustaining the CSCs niche is also gaining substantial importance in promoting resistance to chemotherapy as an extrinsic factor[101]. The TME shapes the morphology and functional features of CSCs, mainly influencing (1) cellular plasticity; (2) hypoxia; (3) metabolic reprogramming; (4) activation of specific signaling pathways; and (5) cell-to-cell interactions[100].

Several *in vitro* studies found that stemness-related genes are overexpressed in HNSCC cell lines resistant mainly to Cisplatin, 5-FU, doxorubicin, and docetaxel. *Sox2*, *Oct4*, *CD44*, *Bmi1*, *ALDH1*, and *Nanog* were the genes most frequently associated with the CSC phenotype. Also, EMT markers (*Slug*, *ZEB1*, *Twist*, *Snail*), as well as drug efflux transporters (ABCG2, ABCC1/ABCC2/ABCC3/ABCC4/ABCC5, ABCB1), epigenetic alterations (HDAC1/HDAC2, SIRT1, KAT6A,/KAT6B), and specific signaling pathways such as Wnt/β-catenin and NOTCH1. These mechanisms endow CSCs to survive against standard cancer therapies and promote tumorigenesis, recurrence, and metastasis after chemotherapy Table 1[99,104-107].

Interestingly, when considering the CSC phenotype and plasticity in chemoresistant HNSCC tumor samples and cell lines, members of the regulator of embryonic stem cell *Sox* and *Oct4* are highlighted over the classical CD44, Bmi1, and even ALDH1 CSCs biomarkers. *Sox2* was associated with clinicopathological parameters of worse outcomes in HNSCC patients and a mediator of therapy resistance *in vitro*. Functionally, *Sox2* induced the expression of the anti-apoptotic protein Bcl-2 and enhanced resistance to apoptosis-inducing agents, including cisplatin[108]. Accordingly, Lee *et al.* found that *Sox2* overexpression was correlated with tumor recurrence and poor prognosis in HNSCC, contributing significantly to the acquisition of stem cell traits *in vitro*[109]. Ectopic expression of *Sox2* in HNSCC cells induced stemness by positive regulation of *Oct4* and *Nanog* and co-expression of CD44. In addition, endogenous levels of *Sox2* were significantly higher in ALDH1 high cells. At the same time, the downregulation of *Sox2* was followed by *Oct4* and *Nanog* down-regulation, decrease in stemness, invasion, EMT mediators, *in vivo* tumorigenicity, and frequency of $CD44^+$ cells. Moreover, *Sox2* enhances the chemoresistance of CSCs to cisplatin, possibly by inhibiting ABCG2 expression and resistance to oxidative stress in $CD44^+$ $CD271^+$ CSCs in HNSCC[109].

Xie *et al.* found that *Sox8* expression was positively associated with chemotherapeutic resistance, higher lymph node metastasis, advanced tumor stage, and shorter overall survival in HNSCC patients[110]. Also, the expression of *Sox8* in cisplatin-resistant HNSCC cell lines is responsible for orchestrating the acquisition of the CSC phenotype *via ABCG2*, *Sox2*, *Oct4*, and *Bmi1* expression but also resistance to therapy and activation of EMT and Wnt/β-catenin pathway, favoring tumor invasion and progression. These findings indicate that *Sox8* could be used as a biomarker and a possible target to eradicate the CSCs and increase tumor response to standard therapies toward HNSCC[110].

Several *in vitro* studies investigating the relevance of CSCs on chemoresistance initially characterize the CSCs subpopulation based on the expression levels of CD44 and ALDH1. Nör *et al.* showed that treatment with low doses of cisplatin promotes *Bmi1* and *Oct44* expression and increases the CSCs fraction identified as $CD44^{high}$ $ALDH^{high}$, indicating that these cells are intrinsically resistant to treatment and can expand after therapy[111]. The study by Chen *et al.* elegantly confirmed that $Bmi1^+$ CSCs are enriched *in vivo* after treatment with cisplatin, being able to reconstitute the tumor heterogeneity and are the main responsible for recurrence[112]. Kulsum *et al.* found that HNSCC cell lines resistant to cisplatin and 5-FU showed enrichment of $CD44^+$ $ALDH1^+$ subpopulation, stemness, expression of *ABCG2*, *Sox2*, *Nanog*, *Oct4*, and *NOTCH1* genes, and G0/G1 or S phase arrest[113]. One of the mechanisms by which the $CD44^{high}/ALDH1^{high}$ cells become resistant may be the upregulation of the DOT1L and monomethyl l-H3K79 that lead to miR-10 activation, resulting in cytoskeleton remodeling *via* RhoC and upregulation of prosurvival molecules such as cIAP-2 and XIAP[114]. Another mechanism associated with cisplatin resistance of $CD44^{high}/ALDH1^{high}$ is the secretion of FGF2. Most importantly, cisplatin combined with FGFR2 inhibition decreased the percentage of $CD44^{high}/ALDH1^{high}$, and no CSCs enrichment was noticed after cisplatin exposure, indicating that blocking FGFR is an attractive target to eliminate the CSCs in HNSCC[115].

Table 1. The main mechanisms involved in chemotherapy resistance of cancer stem cells in HNSCC

Author	Type of study	Drug and concentration	Cell line	CSC isolation	Associated genes	Main findings
Oliveira *et al.*[96]	*In vitro*	Cisplatin (9-92 μM)	CAL-27 CisR and SCC-9CRR	$ALDH1^+$ $CD44^+$	*HDAC1,HDAC2, SIRT1, KAT6A, KAT6B, ZEB1, Bmi1*	• The mRNA levels of *HDAC1*, *HDAC2*, *SIRT1*, *KAT6A*, and *KAT6B* were up-regulated in cisplatin-resistant cell lines, indicating activation of epigenetic mechanisms for chemoresistance acquisition • Activation of EMT program via association of epigenetic regulators and *ZEB1* is involved with resistance to cisplatin • CSC subpopulation increased in cell lines with increasing levels of cisplatin resistance, which was also associated with high expression of Bmi1
Lee *et al.*[109]	*In vitro, in vivo*	Cisplatin (5- 50 μM)	SNU1041 and FaDu	$ALDH1^{high}$ $CD44^+$	*Oct4, Sox2 Nanog, Twist, Snail, Slug*	• *SOX2* overexpression is associated with recurrence and contributes significantly to acquiring stem cell traits in HNSCC cell lines • SOX2 expression is high in $ALDH1^{high}$ $CD44^+$cells, and its down-regulation was followed by *Oct4* and *Nanog* down-regulation, decrease in stemness, invasion, EMT, and frequency of $CD44^+$ cells • SOX2 contributes to the resistance of CSCs to cisplatin, and its inhibition decreases CSCs viability, possibly by the inhibition of ABCG2. • Downregulation of ABCG2 in CSCs overexpressing SOX2 restored drug sensitivity after cisplatin treatment
Xie *et al.*[110]	*In vitro, in vivo*	Cisplatin (1-10 μM)	SCC9-res cells CAL27-res	$CD44^+$ $CD24^-$	*Oct4*, *Sox2*, *Bmi1*, *SOX8*, *ABCG2*	• Cisplatin-resistant HNSCC cell lines acquire CSCs properties, characterized by increased *Oct4, Sox2, Bmi1, and ABCG2* expression, self-renewal potential, EMT activation, and tumorigenesis *in vivo*, which was mediated by *SOX8* upregulation • *SOX8* knockdown decreases the expression of CSCs associated genes as well as *ABCG2* and inhibits sphere formation, $CD44^+$ $CD24^-$ fraction, migration, and invasion in cisplatin-resistant cell lines • *EMT* was successfully reversed after *SOX8* knockdown and inhibited metastasis • Moreover, *SOX8* knockdown repressed tumor metastasis mainly due to inhibition of the Wnt/ β-catenin signaling pathway through the transcriptional regulation of *FZD7*
Nör *et al.*[111]	*In vitro, in vivo*	Cisplatin (different concentrations)	UM-SCC-1, UM-SCC-22A, and UM-SCC-22B	$ALDH^{high}$ $CD44^{high}$	*Bmi1*, *Oct4*	• Exposure to 2μM cisplatin for 24h showed no impact on cell survival in malignant cells. However, when sorted $ALDH^{high}CD44^{high}$ cells were exposed, cisplatin doubled the CSCs fraction • Low concentrations of cisplatin-induced the expression of *Bmi1* and *Oct4* genes, CD44, and orosphere formation in unsorted and sorted CSCs, indicating that this therapy contributes to the acquisition and maintenance of stemness
Chen *et al.*[112]	*In vivo*	Cisplatin (1mg/Kg body weight)	SCC1, SCC1R, SCC9, SCC22B, SCC23, SCC23R, HN13	$Bmi1^+$ $EpCAM^+$ (primary mouse	-	• Bmi1 identifies a population of CSCs responsible for HNSCC initiation, progression, and metastasis using an elegant *in vivo* model

				HNSCC) ALDH^{high} CD44^{+} EpCAM^{+} (Primary human HNSCC)		of genetic lineage tracing Bmi1^{+} CSCs are located in lymph nodes and in the invasive front of HNSCC, mediating invasive behavior and metastasis ● Bmi1^{+} CSCs are enriched after *in vivo* treatment with cisplatin and were able to reconstitute the tumor heterogeneity after therapy, indicating that these cells are one of the major causes of recurrence ● Targeting Bmi1^{+} CSCs with Bmi1 or AP-1 inhibitors and the tumor bulk with cisplatin resulted in improved therapeutic outcomes, reduced tumor size, and the incidence of lymph node metastasis *in vivo*
Kulsum *et al.*[113]	*In vitro, in vivo*	Cisplatin (2-32 μM), Docetaxel (2-15nM) and 5-FU (5-100μM)	Hep-2, Hep-2 CisR, Cal-27, Cal-27 CisR, Cal-27 5FUR, Cal-27 Dox	CD44^{+}, CD133^{+}, ALDH1A1^{+}	*Oct4, Sox2, Nanog, CD44, NOTCH1, CD133, ALDH1A1, ABCG2*	● Cell lines resistant to cisplatin and 5-FU showed enrichment of CD44^{+}, CD133^{+} and ALDH1A1^{+} CSCs, increased expression of *ABCG2*, *Sox2*, *Nanog*, *Oct4*, and *NOTCH1* genes, and cell cycle dysregulation, characterized by G0/G1 or S phase arrest ● Increased spheroid formation and migration were also observed in resistant cell lines ● *Oct4*, *Sox2*, and *Nanog* expression represent driving forces behind the induction of drug-induced chemoresistance in HNSCC ● Depletion of ALDH1A1 with small molecule inhibitor (NCT-501) in resistant cell lines inhibited tumor burden *in vivo* and increased the efficacy of cisplatin in patient-derived *ex vivo* explant
Bourguignon *et al.*[114]	*In vitro*	Cisplatin (different concentrations)	HSC-3	ALDH^{high} CD44^{high}	*Oct4, Sox2, Nanog*	● HA (matrix hyaluronan) promotes aggressiveness in highly tumorigenic ALDH^{high} CD44^{high} tumor cells ● Up-regulation of *DOT1L* and monomethyl I-H3K79 lead to miR-10 production in HA-treated CSCs ● miR-10 increases the cytoskeleton regulator RhoC in CSCs and *DOTL1* signaling inhibition via DOT1L siRNA or anti-miR-10b inhibitor decreases RhoC, tumor cell migration/invasion, expression of survival proteins (cIAP-2 and XIAP) and contributes to increasing chemosensitivity ● Inhibition of cIAP-2 or XIAP expression enhances cisplatin-induced chemosensitivity in ALDH^{high} CD44^{high} CSCs ● Taken together, *DOTL1* and miR-10 are important targets for future therapies to decrease stemness, induce CSCs death and increase its susceptibility to standard chemotherapy
McDermott *et al.*[115]	*In vitro, in vivo*	Cisplatin (2 μM)	UM-SCC-1 and UM-SCC-22B	ALDH^{high} CD44^{high}	-	● *FGF2* and *EREG* mRNA were increased in cisplatin-treated ALDH^{high} CD44^{high} ● TNFα, IFNγ, IL-6, and NF-κB signaling pathways were associated with cisplatin resistance in ALDH^{high} CD44^{high} cells ● FGFR1-4 inhibition, together with cisplatin treatment, promoted a 50% reduction in ALDH^{high} CD44^{high} ● After *FGFR2* knockdown, cisplatin no longer increased the ALDH^{high} CD44^{high} CSC in HNSCC cell lines ● Therapeutic inhibition of FGFR might contribute to eliminating ALDH^{high} CD44^{high} cisplatin-resistant CSCs
Elkashty *et al.*[117]	*In vitro, in vivo*	Cisplatin (0.817 μg/mL) 5-FU (3.644 μg/mL)	SCC12 and SCC38	CD44^{+}CD271^{+}	*Oct4, Sox2*	● CD44^{+} CD271^{+} cells showed increased resistance to oxidative stress in HNSCC (which is a cytotoxic effect of cisplatin) and higher expression of *Bmi1*, *Oct4*, *Sox2*, *SMO*, and *GLI1* genes after exposure

						to cisplatin and 5-FU
Yu *et al.*[119]	*In vitro, in vivo*	Cisplatin (6.25-100 μM), 5-FU (6.25-100 μM) and doxorubicin (1.25-20 μM)	OECM1-SP SCC25-SP	Side Population (SP)	*CD133, ABCG2, ALDH1A1*	• CD133 was significantly up-regulated in SP cells, which also demonstrated high chemoresistance and expression of ABCG2 • Depletion of CD133 was associated with decreased SP frequency and attenuated *in vivo* tumor formation • Targeting CD133 together with cisplatin treatment abrogated the proliferation of SP cells in HNSCC, indicating that CD133 is a promising therapeutic target to overcome drug resistance in CSCs
Moon *et al.*[120]	*In vitro, in vivo*	Cisplatin (5-100 μM)	YD8, SNU1041, KU-SCC1 and KU-SCC3	$CD44^+$	*Slug*	• CD44+ cells showed high expression of Slug and were significantly resistant to cisplatin, which was also associated with an elevated expression of ABC transporters
Koo *et al.*[122]	*In vitro, in vivo*	Cisplatin (5-50 μM)	HNSCC cell lines (FaDu, SNU1041, SNU1076, YD15, SCC25, and HN6) and three HNSCC CSCs cell lines (K3, K4, and K5)	*Oct4* overexpression	*SOX2* *Nanog*	• Oct4 overexpressing cells in differentiated HNSCC cell lines can drive the acquisition of stem-like phenotype • Oct4 overexpressing cells were more resistant to cisplatin, which was associated with increased expression of ABCC6, indicating that Oct4 is involved in drug resistance
Ota *et al.*[126]	*In vitro, in vivo*	Cisplatin (1μM)	SAS and HSC-4	*Snail* overexpression	*Oct4, Sox2, Nanog, Bmi1, ABCG2*	• *Snail* overexpression led to increased expression levels of CD44 and ALDH1 as well as in the expression of *Bmi1*, *Nanog*, *Oct4*, *Sox2,* and *ABCG2* genes • EMT was induced in *Snail* overexpressing cells, which was also associated with increased stemness and enhancement of chemoresistance • *in vivo, Snail* overexpression induced an invasive phenotype in non-invasive SAS and HSC-4 cells
Garcia-Mayea *et al.*[135]	*In vitro*	Cisplatin and 5-FU (IC50 or higher concentrations)	HTB-43, CCL-138, and JHU029 and their respective cisplatin-resistant cell lines, SCC25	Growing cells in non-adherent conditions for 3 generations	*Sox2, CD44, ALDH1A1, KLF4, ABCB1, Twist*	• CSCs derived from spheres were more resistant to cisplatin and 5-FU when compared with the parental cells • Cells with higher resistance to cisplatin showed a higher percentage of CSCs • CSCs demonstrated higher levels of LC3II/I, indicating that autophagy may be involved with CSCs resistance to cisplatin
Garcia-Mayea *et al.*[136]	*In vitro, in vivo*	Cisplatin (0-150 μM) Dasatinib (0-3 μM)	HTB-43, CCL-138 and JHU029 and their respective cisplatin-resistant cell lines	Growing cells in non-adherent conditions for 3 generations	*TSPAN1*	• CSCs and cisplatin resistant HNSCC overexpress the *TSPAN1* gene and protein • *in vitro, TSPAN1* inhibition decreased autophagy and EMT traits, induced apoptosis, increased sensibility to chemotherapy and inhibited the pSrc-signaling cascade • *in vivo, TSPAN1* depletion impaired tumor growth and metastasis spreading
Mir *et al.*[138]	*In vitro, in vivo*	Cisplatin (0-150 μM), Desatinib (5-100nM)	Fadu, CCL-138, CCL-138 CisR, JHU-027, SCC-25, HTB-43	Growing cells in non-adherent conditions for 3 generations	*SDCBP*	• Cisplatin resistant cells and CSCs showed high *SDCBP* levels and formed slow-growing but highly aggressive tumors *in vivo* • *SDCBP* inhibition promoted cisplatin sensitization in HNSCC cell lines with high resistance to cisplatin, reduced tumorsphere formation, EMT traits, and CSCs fraction identified as SP • p-Src was identified as a major downstream target in SDCBP-mediated CSC properties and cisplatin resistance in HNSCC • SDCBP protein expression in HNSCC was associated with advanced tumor stage, shorter disease-free survival and overall

						survival
Lee *et al.*[139]	*In vitro, in vivo*	Cisplatin (5-50 μM)	SNU-1041, FaDu, HNSCC CSCs cell lines (K1 and K3)	-	*ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, nuclear β-catenin target genes (cyclin D1, cyclin A, Cyclin E and c-Myc)* *Oct4, Sox2, Nanog, CD44, ABCB1, ABCG2* *Wnt 3a, Wnt 5a Wnt 7a, Wnt 10a Wnt 10b, Wnt 13* *FZD2, FZD4* *FZD5*	● Wnt/β-catenin signaling pathway is activated in CSCs cell lines and β-catenin overexpression drives the acquisition of CSCs properties as self-renewal, stem cell marker expression, including *Oct4*, and chemoresistance ● β-catenin directly regulates *Oct4* transcription in CSCs and *Oct4* overexpression abrogates the inhibition of stemness caused by β-catenin knockdown in CSCs ● Wnt/β-catenin axis mediates the self-renewal of CSCs in HNSCC ● Novel therapeutic strategies for targeting CSCs in HNSCC may focus on the blockade of the Wnt/β-catenin signaling pathway
Byun *et al.*[140]	*In vitro, in vivo*	Cisplatin (different concentrations)	SCC-15, SCC-25, fresh HNSCC	$CD44^+$	-	● $CD44^+$ cells were more resistant to chemo and radiotherapy than $CD44^-$ cells *in vitro* and *in vivo* ● *in vivo* treatment with cisplatin and radiation increased tumor hypoxia, HIF-1α and the fraction of $CD44^+$ cells ● HIF-1α promotes stemness via upregulation of NOTCH1 in HNSCC ● HIF-1α or NOTCH1 knockdown increases susceptibility to cisplatin and radiation, which was mediated by Blc-2 inhibition and caspase-3 expression ● Blocking HIF-1α associated with cisplatin substantially decreased tumor growth *in vivo* ● HIF-1α/NOTCH1 signaling in CSCs can be targeted to impair tumor growth and progression as well as to overcome therapeutic resistance

ABCB1: ATP binding cassette subfamily B member 1; ABCC1: ATP binding cassette subfamily C member 1; ABCC2: ATP-binding cassette sub-family C member 2; ABCC3: ATP binding cassette subfamily C member 3; ABCC4: ATP-binding cassette sub-family C member 4; ABCC5: ATP-binding cassette sub-family C member 5; ABCG2: ATP-binding cassette super-family G member 2; ALDH1A: Aldehyde dehydrogenase 1 family, member A1; *Bmi1*: B lymphoma Mo-MLV insertion region 1 homolog; cIAP-2: Cellular inhibitor of apoptosis 2; DOT1L: DOT1 like histone lysine methyl transferase; EGFR: epidermal growth factor receptor; EMT: epithelial mesenchymal transition; FGF2: fibroblast growth factor; FGFR2: fibroblast growth factor receptor 2; GLI1: glioma-associated oncogene; HDAC1: histone deacetylase 1; HDAC2: histone deacetylase 2; HNSCC: head and neck squamous cell carcinoma; KAT6A: Klysine acetyltransferase 6A; KAT6B: Klysine acetyltransferase 6B; KLF4: kruppel-like factor 4; NOTCH1: neurogenic locus notch homolog protein 1; Oct4: octamer-binding transcription factor, OSCC: oral squamous cell carcinoma; SDCBP: syndecan-binding protein; SIRT1: sirtuin 1; SMO: smoothened, frizzled class receptor; Sox2: sex-determining region Y [SRY]-box; Sox8: SRY-box transcription factor 8; TSPAN1: tetraspanin-1; ZEB1: Zinc finger E-box-binding homeobox 1XIAP = X-Linked Inhibitor of apoptosis.

CD44 is frequently associated with other potential markers of CSC aiming for efficient enrichment of this subpopulation within HNSCC cell lines and tissues. Galbiatti-Dias *et al.* identified the CSC profile of HNSCC cell lines as $CD44^{high}$ $CD133^{high}$ $CD117^{high}$ profile[116]. This CSCs subpopulation demonstrated higher migration capacity and more resistance to Paclitaxel chemotherapy, in addition to an up-regulation of CD44 and down-regulation of *EGFR* transcripts in the HN13 oral cancer cell line[116]. Elkashty *et al.* combined the positivity of CD44 to CD271 (p75NTR), a described marker of CSC in many tumors[117], to isolate an enriched subpopulation of CSCs, followed by their characterization *in vitro*, *in vivo*, and HNSCC tissue samples. The authors found that $CD44^+$ $CD271^+$ cells exhibited higher cell proliferation, sphere/colony formation, chemoresistance to cisplatin and 5-FU, and radioresistance, upregulation of CSCs-related genes (*Sox2*, *Oct4*, *Bmi1*, *Smo*, and *GLI1*), and *in vivo* tumorigenicity[117]. These combined cell markers also showed increased expression in patients with advanced disease.

A study from Oh *et al.* demonstrated that $CD44^+$ cells derived from primary HNSCC had increased expression of ABCG2 and enriched side population[118]. Yu *et al.* found that side population cells characterized by the $CD133^+$ phenotype show elevated chemoresistance and ABCG2 expression, which was abrogated by combining cisplatin with CD133-targeted therapy[119]. Moreover, *Snail* is overexpressed in $CD44^+$ CSCs and associated with cisplatin resistance and high expression of ABC transporters[120]. Interestingly, the percentage of *Oct4* positive cells increases significantly after treatment with 5-FU, cisplatin, and paclitaxel[121], and increased expression of ABCC6 was associated with increased resistance to cisplatin in *Oct4* overexpressing cells, indicating that this poor explored ABC transporter may be relevant to resistance acquisition in HNSCC[122]. Thus, constitutive or acquisition of stem cell and EMT-associated genes are involved with the up-regulation of drug transporters pumps and multi-drug resistance.

The process of EMT is tightly linked with the CSC's biology and chemoresistance in HNSCC. CSCs keep their EMT phenotype until depositing in the distant sites of metastasis (a migratory phenotype), where they change their phenotype toward attaining a MET morphology to proliferate rapidly, causing tumor outgrowth (a proliferative phenotype)[123]. This rapid cellular proliferation leads to hypoxia in the nearby milieu, thereby exacerbating tumor resistance to therapy[124]. Masui *et al.* observed that the CSC-like phenotype is induced after *Snail*-overexpression and is associated with increased $CD44^+/ALDH^+$ in HNSCC cell lines[125]. The EMT and CSC phenotype acquisition in *Snail* overexpressing cells also decreased chemosensitivity. Similarly, Ota *et al.* demonstrated that *Snail*-induced EMT was associated with increased stemness, inducing *in vivo* cancer invasive progression and enhancement of chemoresistance[126]. A recent study from Oliveira *et al.* demonstrated that the CSC subpopulation and activation of the EMT program, characterized by down-regulation of E-cadherin and up-regulation of vimentin, mainly *via* association of epigenetic regulators and ZEB1, is involved with resistance to cisplatin in HNSCC cell lines[96].

It is worth mentioning that the ability of tumor cells to dynamically adapt to signals provided by the tumor microenvironment and/or induced in response to therapy is obtained by the property of cell plasticity at different stages of tumor progression. Cancer cell plasticity reflects genetic and epigenetic alterations in tumor cells, promoting phenotypical diversity and contributing to intra-tumor heterogeneity[127]. EMT and CSCs states are the two most studied axes of tumor cell plasticity and are often tacitly assumed to be synonymous[128]. This is because both cell plasticity axes appear to drive one another *in silico*, *in vitro*, and *in vivo* studies[129]. Notably, both mathematical modeling studies and experimental observations have reported that EMT is also not a unidirectional process since there are one or more hybrid epithelial/ mesenchymal (E/M) states between the two extremes of pure epithelial or pure mesenchymal phenotypes[130,131] during EMT. For this reason, the term Epithelial-Mesenchymal Plasticity (EMP) has been used as a more accurate description of the process.

The same is true for CSC since there may be subsets of CSCs defined as epithelial, mesenchymal, and hybrid E/M (E-CSCs, M-CSCs, H-CSCs)[132,133]. According to Sahoo *et al.* 2022[128], the emerging evidence points to EMT and stemness being semi-independent axes, i.e., not every cell undergoing EMT may acquire stemness and not every cell switching to be a CSC is mandated to show one or more features of EMT. These authors recently proposed a mathematical model to understand the interconnectivity between the EMP and stemness axes aiming to elucidate the critical cellular processes driving metastasis. This model allows many possible couplings between EMP and stemness, showing that all phenotypes - epithelial, mesenchymal, and hybrid E/M - have the potential to be stem-like; however, this potential is likely to be maximum for hybrid E/M cells[128]. On the other hand, tumor cells exhibiting an amoeboid phenotype belong to the utterly

mesenchymal end of the EMP spectrum but show high stemness and metastatic potential[134]. So, many stem cell phenotypes exist across the EMP spectrum that would only be identified based on single-cell RNA sequencing approaches[128].

Garcia-Mayea *et al.* showed that CSCs isolated by sphere formation in non-adherent conditions were more resistant to cisplatin and 5-FU, possibly due to the increased levels of LC3II/I, indicating that autophagy may be involved in within-drug resistance of CSCs[135]. Recently, using the same CSC model, these authors identified by RNAseq the *TSPAN1* (Tetraspanin 1) gene as an essential modulator of chemoresistance in HNSCC[136]. Blocking *TSPAN1* demonstrated encouraging *in vivo* results, leading to impaired tumor growth, EMT acquisition, and metastasis spreading. Another possible target to eliminate HNSCC CSCs and cisplatin resistance is the SDCBP (Syndecan-binding protein), a central contributor in different phases of the metastasis cascade[137,138]. Upon fibronectin and extracellular molecule engagement, SDCBP, as an adaptor protein, interacts with Src and forms a stable complex with FAK in the cellular membrane leading to long-term Src activation. As a result, downstream target signaling pathways such as NF-kB and TGF-β are activated, promoting EMT, tumor migration, invasion, metastasis, and cisplatin resistance[138]. Lee *et al.* showed that Wnt/β-catenin signaling is activated in CSCs, and β-catenin overexpression drives the acquisition of CSCs properties as self-renewal, stem cell marker expression, including *Oct4*, and chemoresistance[139]. In hypoxic conditions, HIF-1α activates NOTCH1, which is responsible for stemness, EMT activation, and resistance to cisplatin in $CD44^+$ cells[140].

All these exposed findings reveal how broad and complex the process of resistance to the chemotherapeutics available today for treatment could be. It also guides us to seek new and innovative drugs focused on CSCs, such as targeted therapy and immunotherapy, for better treatment and prognosis of HNSCC patients. Notably, the plasticity of CSCs must also be considered since their dynamic phenotype switch may be responsible for different levels of resistance even in the same tumor type. As pointed out by Biddle & Marles[141], an effective biomarker should be precise in correlating the presence of phenotypically plastic CSCs with tumor aggressiveness and therapeutic resistance. It would allow more accurate clinical decisions, such as neck dissection and chemotherapy regimens in HNSCC. More recent evidence highlights some meaningful advances, for example, monoclonal antibody therapy anti-CD44v6 and other markers related to EMT signaling pathways activation, such as the Notch, WNT, and ERK/ MAPK pathways. Although, in terms of clinical safety, targeting CSC-specific processes is not well established yet.

CONCLUDING REMARKS

The presence of CSCs in HNSCC and other solid tumors is associated with tumor heterogeneity and resistance to standard therapies. Target CSCs therapy is very challenging as these cells are a dynamic and plastic population capable of switching between different phenotypes and activation states according to the stimuli provided by the TME. As a result, the frequency of CSCs and their spatial localization in the primary tumor and metastatic foci may be variable, leading to different levels of tumor resistance after treatment. Many studies demonstrated that after radio and chemotherapy, CSCs are enriched and guide tumor recurrence and progression.

In this scenario, it is mandatory to characterize the CSCs and their mechanisms of interactions with the TME in HNSCC to better design targeted therapies that efficiently eliminate these cells in combination with standard treatment and/or immunotherapy. Disrupting the TME can lead to hypoxia inhibition and disturb the CSC niche, facilitating CSCs sensitization to chemotherapy. Moreover, CSCs interaction with different cell types in the TME may be impaired, facilitating its elimination and response to standard treatment. It is essential to highlight that CSCs have an efficient drug efflux machinery that should be considered as

possible targets to improve drug accumulation within this subpopulation of tumor cells. Targeting signaling pathways involved with acquiring stemness, such as the Wnt/β-catenin, FGF, and NOTCH1 in HNSCC, may also be an attractive strategy to eliminate the CSCs and drug resistance. Taken together, CSCs are a relevant target to achieve control of disease and treatment response in HNSCC as they represent significant drivers of tumor resistance. Future studies, especially those using cutting-edge methodologies such as scRNAseq, will help to identify new CSCs targets and cellular interactions that can be used to develop new multi-faceted adjuvant therapies.

DECLARATIONS

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Authors' contributions

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Performed data acquisition, as well as provided administrative, technical, and material support: Nunes FD, Rodini CO, Rodrigues MFSD

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Cancer Drug Resistance

Review

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Resistance to energy metabolism - targeted therapy of AML cells residual in the bone marrow microenvironment

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Abstract

In response to the changing availability of nutrients and oxygen in the bone marrow microenvironment, acute myeloid leukemia (AML) cells continuously adjust their metabolic state. To meet the biochemical demands of their increased proliferation, AML cells strongly depend on mitochondrial oxidative phosphorylation (OXPHOS). Recent data indicate that a subset of AML cells remains quiescent and survives through metabolic activation of fatty acid oxidation (FAO), which causes uncoupling of mitochondrial OXPHOS and facilitates chemoresistance. For targeting these metabolic vulnerabilities of AML cells, inhibitors of OXPHOS and FAO have been developed and investigated for their therapeutic potential. Recent experimental and clinical evidence has revealed that drug-resistant AML cells and leukemic stem cells rewire metabolic pathways through interaction with BM stromal cells, enabling them to acquire resistance against OXPHOS and FAO inhibitors. These acquired resistance mechanisms compensate for the metabolic targeting by inhibitors. Several chemotherapy/targeted therapy regimens in combination with OXPHOS and FAO inhibitors are under development to target these compensatory pathways.

Keywords: Bone marrow microenvironment, acute myeloid leukemia, mitochondria, oxidative phosphorylation, fatty acid oxidation, energy metabolism

INTRODUCTION

Acute myeloid leukemia (AML) comprises a highly aggressive, biologically heterogeneous group of hematopoietic disorders involving one or more cytogenetically or molecularly abnormal cell clones. It is primarily a disease of older adults. The standard of care for relapsed and refractory AML has progressed minimally in the past 30 years, with survival rates of less than 12% for patients over 65 years old[1]. Thus, novel therapeutic strategies that are more effective and carry a lower risk of organ damage than current treatments are urgently needed.

AML cells always face two major metabolic challenges: their high rate of proliferation imposes increased bioenergetic demands, and fluctuations in the availability of external nutrients and oxygen in the bone marrow (BM) microenvironment threaten cellular survival. In the BM, AML cells constantly modulate their metabolic state to adapt to this fluctuating microenvironment, shifting between quiescent, proliferative, and differentiated states[2-4]. Highly proliferative AML cells, drug-resistant AML cells, and leukemia stem cells (LSCs) that remain quiescent have been shown to depend on oxidative phosphorylation (OXPHOS). LSCs differ from bulk leukemia cells in that they possess stem cell characteristics including abnormal self-renewal capacity and drug resistance. Persistence of LSCs in the BM microenvironment after chemotherapy is considered an important factor in AML relapse[5]. Both quiescent AML cells and LSCs survive through metabolic activation of fatty acid oxidation (FAO) along with OXPHOS in their mitochondria. Hence, the reprogramming of energy metabolism processes in AML cells is recognized as a potential therapeutic target. Inhibition of OXPHOS and FAO can disrupt metabolic homeostasis, increase reactive oxygen species (ROS) production, and cause apoptosis in AML cells[2,6,7]. However, inhibition of this altered energy metabolism triggers various adaptive mechanisms in AML cells through their interaction with BM stromal cells. Thus, the BM microenvironment provides a setting in which secondary resistance to OXPHOS inhibition can develop, thereby contributing to the survival of AML cells. Therefore, strategies combining chemotherapy and specific molecular targeted therapy may have promise for eliminating BM-resident AML cells and LSCs.

In this review, we summarize the current state of knowledge about mitochondrial OXPHOS and fatty acid metabolism in residual AML cells in the BM microenvironment. We further describe the molecular mechanism by which AML cells acquire resistance to OXPHOS and FAO inhibitors. Finally, we evaluate potential therapeutic regimens combining OXPHOS and FAO inhibitors to target the metabolic vulnerabilities of BM-resident chemoresistant leukemia cells and LSCs.

MAIN TEXT

The BM microenvironment reprograms OXPHOS in AML cells

AML cells' dependence on OXPHOS in the BM microenvironment

Whereas circulating AML cells are effectively eliminated by drug treatment, AML cells residing in the BM acquire resistance to chemotherapy. The BM microenvironment provides growth factors for leukemic cells, promotes immunosuppression, and supports leukemic cell survival. In response, leukemia cells adapt their metabolic state to this constantly changing environment[7-9].

Energy metabolism encompasses the molecular pathways whose products are involved in cellular energy production in the form of ATP. In leukemic cells, energy metabolism relies on OXPHOS and associated

catabolic pathways, including glycolysis and fatty acid metabolism. The energy required for ATP production is produced by the mitochondrial potential, which causes protons to reenter the mitochondria through complex V. Fatty acid metabolism also supplies acetyl-CoA to the tricarboxylic acid (TCA) cycle through FAO [Figure 1].

Recently, the molecular mechanisms by which AML cells undergo metabolic reprogramming and those underlying the antileukemic efficacy of OXPHOS inhibitors have been demonstrated[7,10]. Actively proliferating AML cells respond to their increased energy and substrate demands via upregulation of OXPHOS, glycolysis, and related biochemical pathways. In turn, their bioenergetic efficacy strongly depends on extrinsic signals from the microenvironment[11].

The leukemic BM microenvironment is generally hypoxic; during disease progression, hypoxic areas in the BM expand[12-14]. Indeed, AML cells so strongly depend on OXPHOS for metabolism that they might cause hypoxia in the BM environment. These hypoxic niches are expanded, in part, through activation of the transcription factor hypoxia-inducible factor 1α (HIF-1α)[12,15]. Transcriptional complexes often include metabolic enzymes, which locally supply substrates and cofactors to these complexes[16]. For this reason, OXPHOS itself and the transcription factors that regulate it are attractive targets for novel therapeutic interventions.

The persistence of LSCs and treatment-resistant AML cells in the BM remains the major cause of failure to eradicate AML. Cancer stem cells were initially identified in AML[17,18] and subsequently validated in solid tumors. Across cancers, cancer stem cells share two important features: they can self-renew and produce differentiated progeny. The OXPHOS-dependent survival mechanism of LSCs is common to several solid tumor stem cells. For example, pancreatic cancer stem cells use OXPHOS for survival by accumulating the transcription coactivator peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), which enhances mitochondrial biogenesis and the oxygen consumption rate and is sensitive to inhibitors of mitochondrial respiration[19]. Reliance on OXPHOS has also been observed in other solid-tumor stem cells, including those in brain[10] and breast cancers[20].

Several recent studies have revealed how AML LSCs exploit OXPHOS[21]. The transcriptional and epigenetic signatures of leukemia-initiating LSCs are largely mutation-independent[22-25]. Instead, rewired cellular metabolism has been increasingly recognized to play a significant role in LSC maintenance and treatment resistance in AML[26]. In LSCs, several components of the electron transport chain (ETC) complexes I and V have been shown to be more abundant than in normal hematopoietic stem cells (HSCs)[27]. Notably, AML LSCs overexpress antiapoptotic BCL-2, which has been shown to regulate ATP/ADP exchange across the mitochondrial membrane by facilitating regulation of voltage-dependent anion channels and adenine nucleotides,[28,29] preventing the loss of coupled mitochondrial respiration during apoptosis[30].

Rewiring of mitochondrial function facilitates AML resistance to OXPHOS inhibition

Understanding the crosstalk between AML cells and their microenvironment is critical to targeting the pathways involved in the metabolic reprogramming of chemoresistant AML cells and LSCs. In preclinical studies, most AML models responded to inhibition of OXPHOS via targeting of ETC complex I[3]. However, several clinical trials have shown that the efficacy of these OXPHOS inhibitors is limited[31,32]. In trials of several types of solid tumors, one putative complex I inhibitor, carboxyamidotriazole, had no clinical benefit[31]. Mouse studies of BAY87-2243, a novel complex I inhibitor, demonstrated antitumor activity and no toxic effects, but the phase I trial was terminated because of unexpected toxic effects[32]. These findings indicate that OXPHOS inhibitors have a narrow therapeutic window and emphasize the need to better

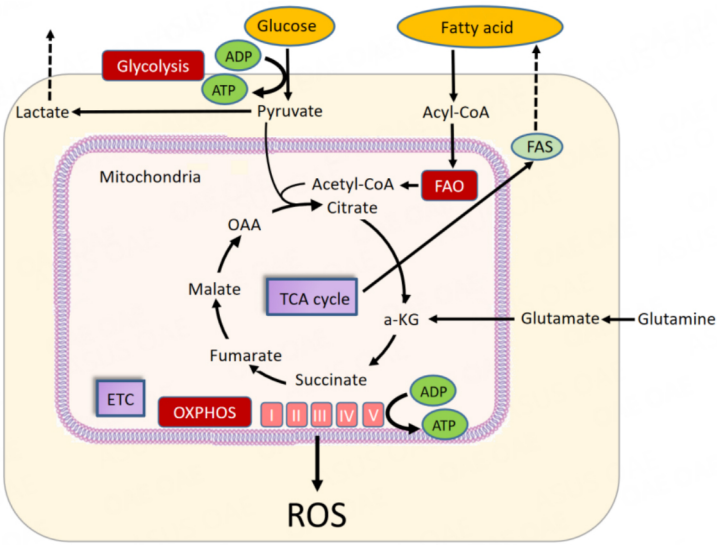

Figure 1. Energy metabolism in AML. Glucose is converted to pyruvate by glycolysis. Pyruvate is converted to acetyl-CoA for use in the tricarboxylic acid (TCA) cycle. ATP is produced by oxidative phosphorylation (OXPHOS) in the TCA cycle and electron transport chain (ETC). Fatty acid metabolism supplies acetyl-CoA to the TCA cycle via β-oxidation of fatty acids (FAO). Glutamine metabolism is the process of converting glutamine to glutamic acid. a-KG: Alpha-ketoglutarate; FAS: fatty acid synthase; OAA: oxaloacetic acid; ROS: reactive oxygen species.

understand how the BM microenvironment enables AML cells to become resistant to the metabolic stress caused by OXPHOS inhibition.

One such mechanism occurring in the tumor microenvironment is the horizontal transfer of mitochondrial DNA from host to tumor cells. Studies using *in vivo* models have shown that this transfer reestablishes respiration and promotes tumorigenesis[33]. In OXPHOS-dependent AML cells, OXPHOS inhibition induced formation of tunneling nanotubes that enabled this mitochondrial DNA trafficking from BM stroma cells to AML cells[34]. In the formation of tunneling nanotubes, a filopodium-like protrusion is extended from one cell to another[35]. This process is positively regulated by activation of motor proteins such as Rho GTPases through actin polymerization[36,37] and filopodia formation through focal adhesion[38]. In addition, a recent study showed that a transmembrane complex gap junction channel opens under ROS-induced oxidative stress via PI3K-Akt activation to enable the transfer of mitochondrial DNA from stromal cells in the BM to HSCs [Figure 2][39].

OXPHOS inhibition-induced horizontal transfer of mitochondria from BM stromal cells to AML cells is accompanied by endogenous mitochondrial fission and elimination of damaged mitochondria by mitophagy, both of which contribute to AML cell survival[34,40]. As the process by which damaged mitochondria are segregated for elimination by autophagy, mitochondrial fission is central to mitophagy[41-44]. Cellular metabolism and cell survival require efficient mitophagy[40,45]. Because of the centrality of these processes in the maintenance of mitochondrial function, both mitochondrial fission and crosstalk with BM stroma cells via tunneling nanotubes may be another mechanism by which AML cells develop resistance to OXPHOS inhibition[46].

LSCs have low rates of energy metabolism and cannot upregulate glycolysis after OXPHOS inhibition. Thus, they are particularly sensitive to OXPHOS blockade[47,48]. However, LSCs are able to maintain their stemness by mitochondrial fission and mitophagy, which balance mitochondrial functions such as energy

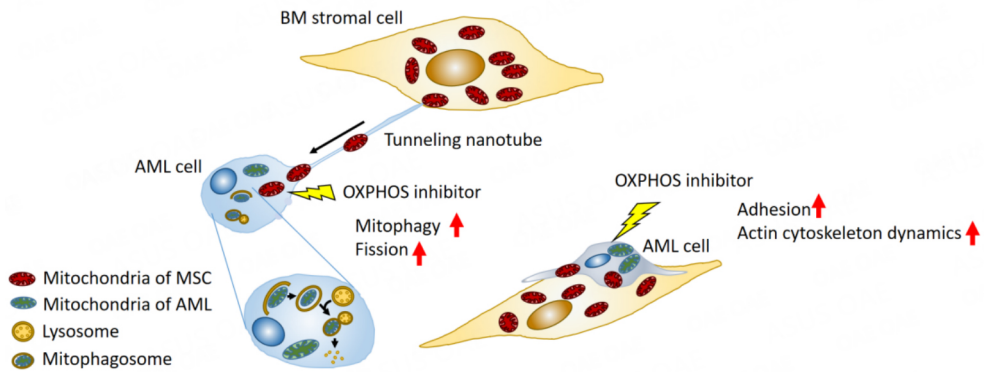

Figure 2. Secondary resistance to oxidative phosphorylation (OXPHOS) inhibition through bone marrow mesenchymal stem cells. OXPHOS inhibition stimulates cell adhesion and actin dynamics in AML cells. The bone marrow (BM) microenvironment facilitates the development of secondary resistance to OXPHOS inhibition by supporting direct mitochondrial trafficking via tunneling nanotubes from BM stromal cells to AML cells. This trafficking is accompanied by mitophagy and mitochondrial fission. MSC: Mesenchymal stem cell.

production, ROS generation, and apoptosis regulation[44]. Given these competing pressures, LSCs have a fragile mitochondrial network. Thus, blocking both OXPHOS and other metabolic pathways is a promising strategy for overcoming OXPHOS resistance associated with the BM microenvironment. Two possible targets include the enzyme ASS1 and the lipid metabolism protein LRP1, both of which are overexpressed in OXPHOS inhibitor-treated AML cells *in vivo*. ASS1 is essential for the biosynthesis of arginine[49], and LRP1 contributes to hemin-induced autophagy in leukemia cells[50,51]. Further investigations will improve our understanding of how these enzymes shape the responses of LSCs to the metabolic and energetic effects of OXPHOS inhibition.

Several repurposed drugs have been shown to inhibit OXPHOS. Biguanides, including metformin, are routinely used for diabetes treatment and have been proposed for use in cancer because they inhibit complex I of the ETC in cancer cells[52]. However, metformin carries a risk of severe lactic acidosis[53,54], which is a safety concern for cancer patients. In addition, because high OXPHOS levels play a key role in cytarabine resistance, treatments combining cytarabine with OXPHOS inhibitors might be more effective than monotherapy with either type of agent[33]. Recently, a novel lipoate analog, devimistat, an inhibitor of two key TCA cycle enzymes, the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes[55,56], showed a satisfactory safety profile in AML patients. In a phase I study, patients with relapsed or refractory AML who received a combination of devimistat with cytarabine and mitoxantrone had a complete remission rate of 50%[57].

In a phase II study of this combination, responses were observed in older patients but not in younger patients. In addition, RNA sequencing analysis showed a decrease in expression of mitochondria-related genes with aging, suggesting that age-related reduction in mitochondrial quality may be related to devimistat response[58]. These are encouraging findings that indicate that this approach would be particularly effective for older patients with the highest unmet medical needs. In sum, the judicious use of novel OXPHOS inhibitors in combination treatments may add to armamentarium of currently available therapeutics.

FAO metabolism of AML cells and LSCs in the fatty acid-abundant BM microenvironment

FAO of AML cells in the BM microenvironment

Adipocytes in the BM microenvironment support the survival of several types of tumor cells by stimulating FAO through fatty acid transfer[59]. While it was reported that BM adipocytes occupy approximately 60% of

the BM in 65-year-old individuals[60], the development of BM adipocytes varies across different skeletal regions, and single-point iliac biopsy may not represent the BM environment of the skeletal system containing the red marrow and yellow marrow. In a previous study, leukemic cells have been shown to colonize in both red and yellow marrow regions, adhere to the cortical bone in the spine, and have enhanced activity in the proximal/distal femur[61]. In addition, radiation therapy accelerates the differentiation of mesenchymal stem cells into adipocytes in BM[62]. Such temporal and spatial changes in the BM microenvironment may play a key role in leukemia's dynamic adaptation of FAO and in leukemia cells' interactions with BM stromal cells.

AML cells generally obtain fatty acids for FAO from the extracellular microenvironment through lipolysis of stored triglycerides[63]. FAO is metabolically activated to promote leukemic cell survival by remodeling and lipolysis of BM adipocytes. FAO is an essential source of mitochondrial NADH and FADH2 for the ETC and provides acetyl-CoA to the TCA cycle to produce ATP[64]. BM adipocytes supply long-chain fatty acids, which are then taken up into the cytoplasm via the scavenger receptor CD36[65,66]. Fatty acids activation is a two-step reaction. In the first step, the fatty acids form acyl-CoA in the cytoplasm. Then, FAO breaks down acyl-CoA to form acetyl-CoA in the mitochondria. Carnitine O-palmitoyltransferase 1 (CPT1) catalyzes a rate-limiting step of FAO; this enzyme conjugates fatty acids to carnitine, which is required for fatty acids to translocate from the cytoplasm to the mitochondria[67]. The internalized fatty acids are further transferred to the AML cell nucleus by the lipid chaperone fatty acid-binding protein 4 (FABP4). In the nucleus, the fatty acids ligate to peroxisome proliferator-activated receptor γ (PPAR)[68]. Activated PPAR induces downstream target genes, including *CD36*, *FABP4*, and the antiapoptotic *BCL2* [Figure 3][69].

As with AML cells, the specific BM microenvironment created by the interaction between LSCs and stromal adipocytes supports the metabolic demands of LSCs. LSCs induce adipocyte lipolysis, which drives FAO in LSCs and facilitates their survival[70,71]. Therefore, CD36 and CPT1 are potential targets for AML. A CD36 neutralizing antibody inhibited metastasis of human melanoma and breast cancer cells[72], and inhibition of CPT1 caused mitochondrial damage leading to cell death in primary AML cells[67].

FABP4 is important in FAO and cancer cell survival in both solid and hematologic cancers. Adipocytes are known to serve as fatty acid reservoirs in breast cancer and melanoma[73,74]. Ovarian cancer cells also survive and proliferate in an adipocyte-rich microenvironment[75]. When primary human omental adipocytes were co-cultured with ovarian cancer cells, the adipocytes underwent lipolysis, and FAO was induced in the cancer cells[63]. These processes are mediated by adipokines including interleukin-8 and by upregulation of FABP4 both in adipocytes and ovarian tumor cells. In studies of leukemia, AML cells co-cultured with BM adipocytes exhibited higher levels of FABP4[69], and knockdown of FABP4 prolonged survival in a mouse model of leukemia[71]. Thus, FABP4's critical role in cancer cell survival involves its interactions with adipocytes.

Activation of β-adrenergic receptors, along with a G protein-coupled cascade that stimulates the lipolytic enzyme hormone-sensitive lipase (HSL), induces lipolysis of adipocytes[76,77]. Ovarian cancer cells have been found to upregulate HSL phosphorylation, thereby stimulating the release of free fatty acids from adipocytes[63]. AML blasts also induce HSL phosphorylation and, thus, activation of lipolysis in BM adipocytes[71].

BM adipocytes also increase AML cells' expression of adiponectin and its downstream target, AMP-activated protein kinase (AMPK), a stress response kinase[69]. AMPK, an important modulator of energy metabolism, is activated upon ATP depletion. Its functions include upregulation of fatty acid uptake, FAO,

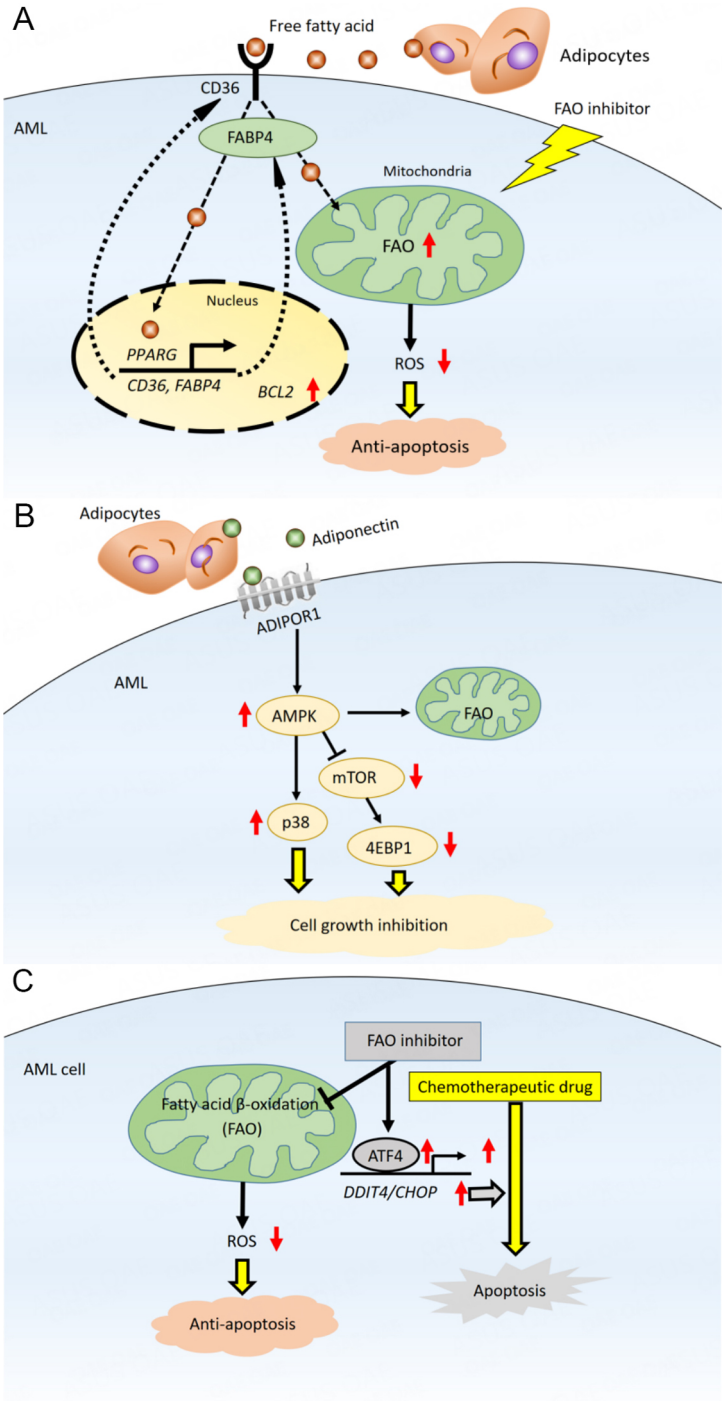

Figure 3. Bone marrow adipocytes promote fatty acid metabolism in AML. (A) Fatty acids derived through lipolysis of stored triglycerides in adipocytes induce upregulation of *PPARG*, *CD36*, and *FABP4* gene transcription, which stimulates fatty acid endocytosis. In mitochondria, fatty acids are metabolized through fatty acid oxidation (FAO), decreasing mitochondrial reactive oxygen species (ROS) formation and intracellular oxidative stress, thereby reducing apoptosis; (B) transcriptional regulation and fatty acid metabolism pathways maintain AML cells in a quiescent state. Activation of AMPK, upregulation of p38 and associated induction of autophagy, and upregulation of antiapoptotic HSP chaperone proteins in this state lead to chemoresistance; (C) in mitochondria, fatty acids are consumed for FAO, resulting in diminished formation of mitochondrial ROS and decreased intracellular oxidative stress. Inhibition of FAO induces an integrated stress response that stimulates transcriptional activation of ATF4 and promotes apoptosis induced by chemotherapy. ADIPOR1: Adiponectin receptor 1; ATF4: activating transcription factor 4; AMPK: AMP-activated protein kinase; FABP4: fatty acid binding protein 4; p38: p38 mitogen-activated protein kinase.

and regulation of autophagy[78,79]. Levels of adiponectin, much of which is supplied by BM adipocytes, have been shown to increase during cancer therapy[80], and to promote chemotherapy resistance in myeloma cells via inducing adipokine secretion of adipokines and activating AMPK-dependent autophagy[81,82]. AMPK also positively regulates responses of an antiapoptotic chaperone heat shock protein that binds to denatured and unfolded proteins and promotes protein refolding or degradation to support AML cell survival[79]. In sum, leukemic cells often rely on fatty acids when they undergo metabolic stress, and the NADH and FADH2 generated by FAO support ATP production, redox homeostasis, biosynthesis, and cell survival.

FAO is involved in the interactions between LSCs and BM stromal cells[83]. LSCs rely on fatty acid uptake and consumption to shape their adaptation to the conditions of the BM microenvironment, their response to drugs, and their development of drug resistance[69]. Several such mechanisms have been identified. Mitochondrial uncoupling in AML cells negatively regulates Bak-dependent mitochondrial permeability transition[84]. In a study using samples from patients with relapsed AML, LSCs acquired the ability to counteract the loss of amino acid metabolism by upregulating FAO[85]. Specifically, this mechanism may underlie the development of resistance to treatment with azacitidine/venetoclax, a common induction regimen used mainly in older patients with AML[85,86]. In addition, a preclinical study demonstrated that cytarabine-resistant AML cells had enhanced FAO and OXPHOS[66]. Thus, targeting the metabolic vulnerabilities of chemoresistant LSCs, such as their dependence on FAO, may be a useful strategy for eradicating these cells.

FAO inhibitors and resistance acquired by compensatory metabolism in the BM microenvironment

FAO inhibition disrupts metabolic homeostasis, increases ROS levels, and induces expression of the integrated stress response mediator ATF4 in AML cells, all of which contribute to apoptosis[87]. Numerous studies have reported the anti-AML effect of inhibition of CPT1, the major rate-limiting enzyme in FAO[67,84,87,88]. CPT1 positively controls FAO by conjugating fatty acids with carnitine to transfer fatty acids into the mitochondrial matrix. Etomoxir is a pharmacological inhibitor of CPT1A, one of the isoforms of CPT1[89], frequently used to block free fatty acids from entering the mitochondria via CPT1. Although the clinical use of etomoxir has ceased because of adverse effects[90], the CPT1 inhibitor perhexiline can sensitize breast cancer cells to paclitaxel[91], and other CPT1 inhibitors[92] are currently being investigated for use in cancer therapy. The CPT1A inhibitor ST1326 has been shown to cause cell growth arrest, mitochondrial damage, and apoptosis in AML cells in a dose- and time-dependent manner[67]. Another novel FAO inhibitor, avocatin B, which is derived from avocados, decreased NADPH levels that were increased by FAO through acetyl-CoA and NADH production, inducing ROS-dependent cell death in AML cells[93,94]. Finally, the fatty acid synthase inhibitor orlistat has induced apoptosis in leukemic cells[2].

The intramitochondrial FAO enzyme very long-chain acyl-CoA dehydrogenase (VLCAD) is critical in supporting both FAO and OXPHOS in AML cells and LSCs. Recently, preclinical studies have demonstrated the antileukemia activity of a novel small-molecule VLCAD inhibitor, a polyhydroxylated fatty alcohol with a terminal alkyne (AYNE)[95]. AYNE reduced mitochondrial respiration by altering FAO, which led to reduced ATP production in AML cells, even though AYNE also moderately upregulated glycolysis. In a mouse model, pharmacological inhibition of VLCAD with AYNE significantly reduced the repopulation potential of leukemia cells and was well tolerated[95]. Notably, normal HSCs compensate for this reduced replicative capacity through glycolysis which maintains their ATP levels and thus their viability[95,96]. These findings demonstrate the importance of focusing on the specific metabolic vulnerabilities of residual AML cells and LSCs that survive chemotherapy-induced stress. Unfortunately, only a few FAO inhibitors have advanced from preclinical to clinical studies [Table 1].

Table 1. FAO inhibitors in clinical trials on cancer treatment

Compounds	Targeted enzyme	Clinical applications	Phase	ClinicalTrials.gov Identifier	Verified
Trimetazidine	3-ketoacyl-C3-ketoacyl-CoA thiolase	Advanced Hepatocellular Carcinoma	Phase 3	NCT03278444	September 2017
Trimetazidine	3-ketoacyl-C3-ketoacyl-CoA thiolase	Intermediate-stage Hepatocellular Carcinoma	Phase 3	NCT03274427	September 2017
Ranolazine	3-ketoacyl-C3-ketoacyl-CoA thiolase	Prostate Cancers	N//A (Pilot Study)	NCT01992016	December 2018

Because AML is heterogeneous and multiclonal, blocking only one part of this complex metabolic system may allow residual cells to adapt metabolically. For instance, it has been shown that BM-derived stromal cells, including adipocytes, diminish the antileukemia effects of FAO inhibitors in AML cells by increasing glycolysis and glucose and free fatty acid uptake[87]. This compensatory induction of glycolysis is a sustained source of ATP to AML cells and, in turn, induces substantial lactate production. Similarly, FAO-deficient *Abcb11*-knockout mice exhibited high FABP4 and CD36 expression and free fatty acid uptake[97]. In sum, it is clear that FAO inhibition initiates several different adaptive mechanisms that promote AML cell survival in the BM microenvironment.

For this reason, treatment options based on combination regimens have been tested. Although FAO inhibition alone can trigger compensatory activation of other metabolic pathways, FAO inhibitors can also synergize with conventional antitumor agents such as paclitaxel[91]. For example, FAO and OXPHOS are increased in cytarabine-resistant AML cells; FAO inhibition with etomoxir induced a metabolic shift from high to low OXPHOS, sensitizing the cells to cytarabine[66]. Similarly, the combination of avocatin B and cytarabine synergized to enhance ROS production and induce apoptosis in AML cells co-cultured with BM adipocytes[87]. The role of avocatin B in apoptosis induction was attributed to activation of endoplasmic reticulum stress-induced ATF4[87]. These findings suggest that AML cells treated with cytarabine exhibit increased dependence on FAO, which may account for the synergism of cytarabine and FAO inhibitors.

CONCLUSIONS

AML cells and LSCs both strongly depend on the production of mitochondrial biomass and on OXPHOS[66] and FAO[84] for survival. Compared to healthy HSCs, AML cells and LSCs are more susceptible to mitochondrial stress because their respiratory chain reserve capacity is lower[98]. These characteristic differences in the metabolism of AML cells and their normal hematopoietic-cell counterparts represent a specific vulnerability of leukemia cells and therefore are drawing a great deal of attention as targets for AML therapy. The results of studies in preclinical models using agents that target fatty acid metabolism have been encouraging.

Although they are vulnerable to the targeting of their metabolic pathways, AML cells, drawing on microenvironmental factors, can adapt to metabolic stress by activating metabolic bypass processes. Several *in vivo* studies and clinical trials have shown that the use of metabolic inhibitors alone is ineffective both because of their narrow therapeutic window and because of these adaptive mechanisms[87]. Alternatively, inhibition of FAO and other metabolic mechanisms along with conventional chemotherapy or targeted therapy may synergistically eradicate chemotherapy-resistant AML cells present in the BM.

In conclusion, understanding AML cell metabolism in the specific context of the BM microenvironment is crucial to improving therapies for AML. Because characteristics of the BM microenvironment enable the acquisition of resistance to OXPHOS and FAO inhibitors, drug combination strategies that interfere with

these adaptations are needed. A more comprehensive understanding of the mechanisms of AML cell metabolism in future studies may reveal new treatment options targeting OXPHOS and FAO, enhance the efficacy of chemotherapeutic agents that target related pathways, reduce the toxicity of these agents, and enable the translation of new combinations of agents into clinically applicable treatment strategies.

DECLARATIONS

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Cancer Drug Resistance

Review

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Targeting regulated cell death pathways in acute myeloid leukemia

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Abstract

The use of the BCL2 inhibitor venetoclax has transformed the management of patients with acute myeloid leukemia (AML) who are ineligible for intensive chemotherapy. By triggering intrinsic apoptosis, the drug is an excellent illustration of how our greater understanding of molecular cell death pathways can be translated into the clinic. Nevertheless, most venetoclax-treated patients will relapse, suggesting the need to target additional regulated cell death pathways. To highlight advances in this strategy, we review the recognized regulated cell death pathways, including apoptosis, necroptosis, ferroptosis and autophagy. Next, we detail the therapeutic opportunities to trigger regulated cell death in AML. Finally, we describe the main drug discovery challenges for regulated cell death inducers and their translation into clinical trials. A better knowledge of the molecular pathways regulating cell death represents a promising strategy to develop new drugs to cure resistant or refractory AML patients, particularly those resistant to intrinsic apoptosis.

Keywords: Acute myeloid leukemia, regulated cell death, apoptosis, ferroptosis, necroptosis, autophagy

INTRODUCTION

Regulated cell death (RCD) is a biologically controlled process that differs from accidental cell death (ACD) by its reliance on defined molecular signaling pathways and tight regulation. Its well-defined nature implies that it can be modulated by pharmacological or genetic interventions contrary to ACD[1-3]. Schweichel and Merker were the first to report the presence of three distinct cell death morphologies: type I (apoptosis), type II cell (cell death associated with autophagy) and type III (necrosis)[4]. While apoptosis is the most well-known RCD, many other pathways and molecular characteristics have subsequently been described[2]. A better understanding of the mechanisms driving RCD may lead to the discovery of new anticancer drugs or the repositioning of older drugs to treat aggressive cancers.

Acute myeloid leukemia (AML) is a severe hematological malignancy driven by various molecular alterations and mainly occurring in adults > 60 years old. Treatment modalities of newly diagnosed AML depend on age, general conditions, comorbidities, and molecular risk factors based on cytogenetics and the presence of molecular alterations. These mutations are integrated together with cytogenetic abnormalities in the widely used and recently updated ELN 2022 classifications[5,6]. More than 60% of younger patients will be cured by an intensive cytotoxic therapy (ICT) induction based on the association of anthracyclin and cytarabine (7 + 3), followed by consolidations with high doses of cytarabine and/or allogeneic stem cell transplantation[7]. Half of the patients > 65 years cannot receive ICT because of age, poor general status, or comorbidities. In this context, the hypomethylating agents (HMA) decitabine and azacytidine (AZA) have been associated with complete response rates of 20-30% and 10-month survival[8,9], highlighting the need for more effective treatments for older and not adequately fit patients.

BCL-2 inhibition is an excellent illustration of how the molecular understanding of RCD has led to the rapid development of a drug that has transformed the therapeutic treatment landscape for older AML patients. Venetoclax (VEN) in combination with the hypomethylating agent AZA has become part of standard frontline therapy for patients not eligible for ICT by improving the rates of response and overall survival compared with AZA monotherapy[10,11]. However, 10% to 50% of newly diagnosed patients with AML will not respond to VEN-AZA. In addition, half of the patients have relapsed by 18 months and no plateau is seen on overall survival curves. In the population of VEN-AZA refractory or relapsed patients, response rate and overall survival are poor (20% and 2.4 months, respectively)[12,13]. The combination of VEN with ICT is also associated with high response, but 3% to 15% of patients do not respond to the treatment[14]. Altogether, these data show that targeting RCD is a valuable strategy and has already improved the efficacy of the current AML therapeutic strategies. However, there is a real need to develop new drugs to go beyond BCL2 inhibition in AML[13]. In this review, we will discuss the main RCD pathways, describe their therapeutic targeting and discuss the main challenges for translating preclinical results into the clinic.

REGULATED CELL DEATH PATHWAYS

Intrinsic apoptotic pathway

Proapoptotic and antiapoptotic members of the BCL2 protein family share one to four BCL2 homology (BH) domains and control intrinsic apoptosis[15,16]. Under physiological conditions, BCL2-associated X (BAX) resides in the cytosol in an inactive conformation while BCL2 antagonist/killer 1 (BAK) is inserted at the outer mitochondrial membrane (OMM) via an α9 helix that connects with voltage-dependent anion channel 2 (VDAC2)[17]. In response to apoptotic stimuli, BAX and BAK associate to form pores in the OMM, inducing mitochondrial outer membrane permeabilization (MOMP, Figure 1).

MOMP is regulated by the members of the BCL2 protein family containing a single BH3 domain named BH3-only proteins. The main representatives of this class are p53-upregulated modulator of apoptosis

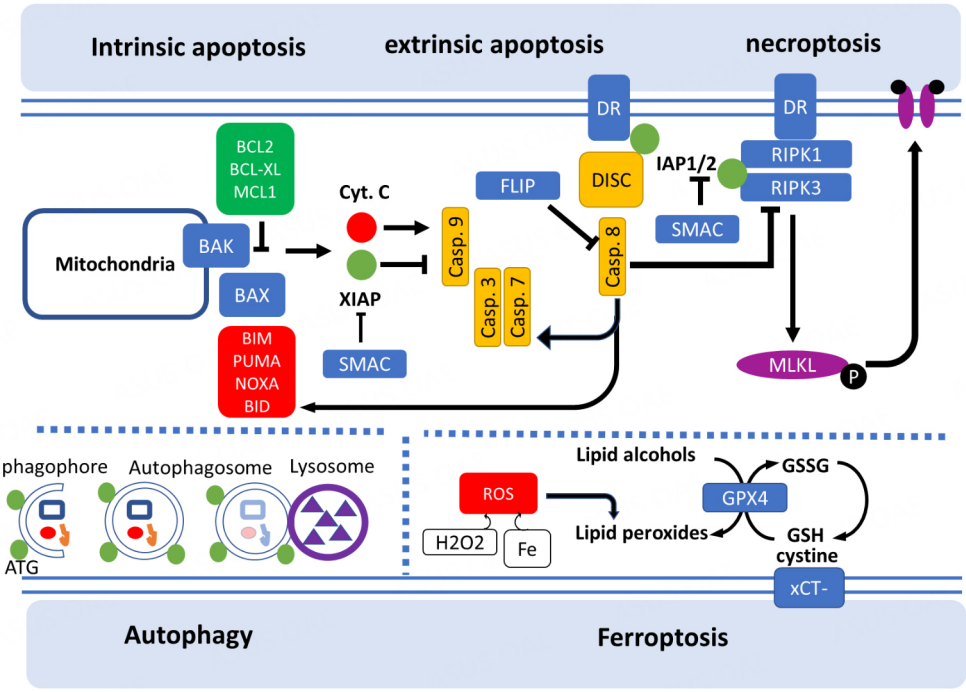

Figure 1. Mechanisms of regulated cell death pathways.

(PUMA), BCL2-like 11 (BCL2L11), also called BCL2-interacting mediator of cell death (BIM), phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1, also called NOXA) and BH3 interacting domain death agonist (BID)[18]. All these proteins have a proapoptotic role by directly interacting with BAX and/or BAK to induce MOMP. Conversely, MOMP is blocked by a series of proteins that have an antiapoptotic role. This includes BCL2, BCL2-like 1 (BCL2L1, also known as BCL-X_L), MCL1, BCL2 like 2 (BCL2L2, also known as BCL-W), and BCL2 related protein A1 (BCL2A1, also known as BFL-1)[19]. Other BH3-only proteins, including BCL2-associated agonist of cell death (BAD), BCL2 modifying factor (BMF), or harakiri, BCL2 interacting protein (HRK), have the ability to induce MOMP in the absence of direct interaction with BAX or BAK by limiting the ability of the antiapoptotic BCL2 family members to sequester BAX BAK, or BH3-only activators[20].

MOMP promotes the cytosolic release of cytochrome c (Cyt c) and diablo IAP-binding mitochondrial protein (DIABLO), also known as second mitochondrial activator of caspases, SMAC[16]. The cytosolic pool of Cyt c binds to apoptotic peptidase activating factor 1 (APAF1) and pro-caspase 9 to form the multiprotein complex called the "apoptosome" responsible for caspase 9 activation. Activated caspase 9 catalyzes the proteolytic activation of the executioner caspase 3 and caspase 7, inducing apoptotic cell death. Blocking post-mitochondrial caspase activation with Z-VAD-fmk and Q-VD-OPh caspase inhibitors delays but does not completely rescue apoptosis *in vitro* and *in vivo* as it induces a switch to other types of RCD[2].

Extrinsic apoptosis

The extrinsic apoptosis pathway is initiated by cell membrane proteins known as death receptors (DR). Proapoptotic death receptors include FAS, also known as APO1 and CD95, the tumor necrosis factor (TNF) receptors TNFR1 and TNFR2 and the TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5. DR ligation allows for the assembly of the "death-inducing signaling complex" (DISC), a multiprotein complex that activates caspase 8. At this point, the execution of extrinsic apoptosis driven by DR follows

two distinct pathways. In most cancer cells, caspase 8-mediated cleavage and activation of BID converges with intrinsic apoptosis by triggering a BAX/BAK-dependent MOMP[21]. However, in some cells, (for instance, mature lymphocytes), the caspase 8-dependent activation of caspase 3 and caspase 7 is sufficient to promote cell death independently of mitochondria and BCL2-family of proteins[22]. In DR-induced apoptotic intrinsic pathway, caspases are the main executioner of cell death, causing rapid proteolysis, DNA fragmentation and chromatin condensation, which are the hallmarks of apoptosis.

Intrinsic and extrinsic apoptosis are both regulated by the class of inhibitors of apoptosis proteins (IAPs). Among the 8 IAP family members described in humans, the best known is XIAP which inhibits caspases 3, 7, and 9[23]. IAP proteins are antagonized by SMAC/DIABLO, which is released by mitochondria during MOMP[24].

Necroptosis

Tumor necrosis factor alpha (TNF-α) is a potent trigger for apoptosis, but it was observed that cells treated with TNF-α and the caspase inhibitor zVAD-fmk were still dying without caspase activation, suggesting the presence of an alternative RCD pathway[25]. In 2005, Degterev et al. coined the term necroptosis by demonstrating that this unique RCD distinct from necrosis and apoptosis[26] is initiation by the receptor-interacting protein RIPK1 and could be inhibited by the pharmacological agent necrostatin-1. Indeed, necroptosis is initiated by the activation of the cell death receptors such as TNFR1 and RIPK1 (if caspase 8 is inactive) and depends on the subsequent activation of RIPK3 and the protein complex termed mixed lineage kinase domain-like pseudokinase (MLKL), resulting in the formation of pore on the cell membrane followed by cell death. Nevertheless, several of the upstream signaling elements of extrinsic apoptosis and necroptosis are shared, and sensitivity to each death pathway is regulated by the same set of regulatory molecules, including FLIP, the cellular inhibitors of apoptosis proteins cIAP1 and SMAC/DIABLO[27].

Ferroptosis

The term ferroptosis was coined in 2012 to describe a cell death that can be triggered by inactivation of the cystine/glutamate antiporter (also known as SLC7A11), leading to depletion of intracellular glutathione (GSH) or direct inhibition of glutathione peroxidase 4 (GPX4). GPX4 inhibition induces an accumulation of reactive oxygen species (ROS), ultimately leading to lipid peroxidation via the iron-dependent Fenton reaction, where H_2O_2 and iron react to generate hydroxyl radicals[28,29]. Inactivation of GPX4 through depletion of GSH with Erastin or with the direct GPX4 inhibitor RSL3 ultimately results in overwhelming lipid peroxidation that can be rescued by the use of antioxidant ferrostatin-1 or liprostatin-1 that block lipid peroxidation[30,31]. Ferroptosis is regulated at several levels, including amino acids, lipids (particularly polyunsaturated fatty acids (PUFA)) and iron metabolism[32-36]. In addition, ferroptosis is regulated by the suppressor protein 1 (FSP1) and dihydroorotate dehydrogenase (DHODH) that reduce ubiquinone (CoQ) to ubiquinol ($CoQH_2$) on the plasma membrane and inner mitochondrial membrane, respectively. $CoQH_2$ acts as a hyperoxide radical-trapping antioxidant and decreases lipid peroxidation, resulting in ferroptosis suppression[37,38]. Finally, GTP cyclohydrolase-1 (GCH1) and its metabolic derivatives tetrahydrobiopterin/dihydrobiopterin (BH_4/BH_2) are also potent antioxidants protecting against ferroptosis[39,40]. Ferroptosis is also regulated by the non-canonical activities of TP53 on cellular metabolism in a context-specific manner[41]. TP53 enhances ferroptosis by inhibiting SLC7A11 expression or increasing expression of spermidine/spermine N1-acetyltransferase 1 (SAT1) and glutaminase 2 (GLS2). Likewise, TP53 can inhibit ferroptosis by reducing dipeptidyl peptidase 4 (DPP4) activity or inducing cyclin-dependent kinase inhibitor 1 A (CDKN1A/p21) expression. Unlike the other RCDs, it remains to be determined whether ferroptosis is a consequence of physiological processes or necessary for development as opposed to only pathophysiological situations[42].

Autophagy

Macroautophagy (hereafter autophagy) is a ubiquitous catabolic process that involves the degradation of cytoplasmic components, including intracellular organelles, via the lysosomal pathway. The first step of autophagy is the formation of phagophores, followed by the generation of double-membrane autophagosomes regulated by a series of well-conserved autophagy-regulated genes (ATG). Late steps involved the fusion of autophagosomes to lysosomes, leading to cargo degradation by lysosomal enzymes and the recycling of intracellular contents providing fuels for cell growth[43]. The autophagic response provides cytoprotective effects, as indicated by the fact that blocking autophagy with pharmacological or genetic interventions generally induces cell death through the accumulation of toxic proteins, damaged organelles or undigested autophagosomes toxic for tumor cells[43]. Autophagy is often activated alongside other RCD, such as ferroptosis, which can be promoted by the autophagic degradation of ferritin, extrinsic apoptosis or necroptosis[2]. The main features of RCD are summarized in Table 1.

Other cell death pathways

Pyroptosis is an inflammatory form of lytic RCD that frequently occurs in response to microbial infection by forming a multiprotein complex termed the inflammasome, which activates caspase 1 and forms Gasdermin D (GSDMD) dependent plasma-membrane pores[44]. Pyroptosis is mechanistically distinct from apoptosis and characterized by the absence of DNA fragmentation and the presence of nuclear condensation coupled with cell swelling. Large bubbles eventually form at the plasma membrane and rupture to expel cellular contents.

Mitotic catastrophe is a physiological mechanism by which the cells avoid aneuploidy and hence decrease tumorigenic potential. Accordingly, induction of mitotic catastrophe both precipitates oncogenesis and constitutes a therapeutic endpoint in cancer cells[45].

Inter-regulation and hierarchy between cell death pathways is complex and not completely understood. For instance, mitotic catastrophe is closely related to apoptosis by their common induction by cellular stress and DNA damage. For this reason, some authors suggest that mitotic catastrophe is not a distinct mechanism of death, but one that can occur through necrosis or apoptosis depending on the molecular profile of the cells[46]. Other examples of cross-talk between RCD include the link between ferroptosis and autophagy, in particular through ferritin degradation or the involvement of lysosomes in iron storage and redistribution[47,48]. Depletion of the ferroptosis trigger GPX4 also sensitizes cells to pyroptosis[49], necroptosis[50] and apoptosis[51]. Apoptosis and necroptosis are also linked through caspase 8 cleavage [Figure 1]. Caspase 8 is also an important player in pyroptosis induction, as it has been shown that genetic lesions in XIAP result in increased inflammation and death-associated caspase-8 and GSDMD processing in diseased tissue[52]. Common stimuli can also result in different RCD depending on the cell type. For instance, blockage of iron transport into the lysosome can induce ferroptosis in breast cancer stem cells, whereas it induces mitochondrial BAX/BAK-dependent cell death in AML models[53,54].

RCD INDUCERS IN AML

Apoptotic inducers

Agents targeting intrinsic pathway

Most chemotherapies and radiotherapies are known to induce apoptosis of cancer cells in response to DNA damage or cellular stress[55]. As a consequence, *in vitro* and *in vivo* evidence indicates that TP53-mutated cells have impaired apoptosis signaling pathways, and these cells are typically less susceptible to cytotoxic chemotherapy[56].

Table 1. Summary of the main regulated cell death mechanisms

Type of RCD	Trigger	Facilitator	Inhibitor	Executioner
Intrinsic apoptosis	Mitochondrial outer membrane permeabilization by BAX/BAK macropores and cytochrome c release	Proapoptotic factors from the BCL2 family	Antiapoptotic factors from the BCL2 family, Inhibitors of apoptosis (IAPs)	Proteolysis by executioner caspases
Extrinsic apoptosis	Activation of membrane cell death receptors	Death-inducing signaling complex (DISC). SMAC/DIABLO	Inhibitor of apoptosis (IAPs), FLIP	Proteolysis by executioner caspases
Necroptosis	Activation of membrane cell death receptors	RIPK1, RIPK3, SMAC/DIABLO	Caspase 8	Plasma membrane disruption by MLKL
Ferroptosis	Inhibition of GPX4, decrease of cystine uptake	hydroxyl radicals generation through Fenton reaction	Glutathione (GSH), antioxidant defense	Lipid peroxidation of plasma and intracellular membranes
Autophagy	Phagophore formation and fusion to the lysosome	Autophagy-related proteins (ATG)	Inhibition of lysosome acidification	Accumulation of toxic proteins or organelles leading to cellular stress

Inhibitors of BCL2 family members

Besides VEN, several newly developed BCL-2 inhibitors are currently in various stages of investigation in AML and other leukemia models [Table 2][13,57-60]. In addition to BCL-2, other members of the BCL-2 family of antiapoptotic proteins (MCL-1, BCL-XL, BFL-1) are the target of small molecules with the goal of inducing BAK/BAX activation and promote intrinsic apoptosis. The antiapoptotic protein MCL-1 plays a critical role in inhibiting BAX/BAK activation. MCL1 dependency on leukemia blasts is associated with resistance to BCL2 inhibition by VEN. Several highly potent direct MCL-1 inhibitors have recently entered preclinical and clinical development [Table 2][61-64]. The therapeutic window of these inhibitors is narrow because of the high expression of MCL1 in cardiac and hepatic tissues[65]. Due to these safety concerns, indirect MCL1 inhibitors are also under evaluation. CDK9 inhibitors are in various stages of evaluation in AML [Table 2][66-73]. Addition of alvocidib to ICT improved response rates but not survival in a recently published phase 2 clinical trial[67]. Novel CDK9 inhibitors are currently in early phase trials [Table 2][74-78]. BCL-XL inhibition by navitoclax has been shown to be active in preclinical AML models[79,80]. Toxicity for platelets limited its clinical development; nevertheless, navitoclax in combination with ICT or targeted therapy is still under evaluation in acute lymphoblastic leukemia[81] or myelofibrosis (TRANSFORM-1, NCT04472598). Navitoclax in combination with VEN and decitabine may be a valuable option for VEN-refractory AML patients (NCT NCT05222984). Finally, BFL1 (BCL2A1) inhibition may also be an interesting option since the recent discovery of specific inhibitors, but the drug has not been specifically tested in leukemia models[82,83].

BAX/BAK activators

BAK and BAX are crucial agents in promoting MOMP through protein oligomerization across the OMM. Recent findings showed direct activation of BAX by BTSA1, a pharmacological BAX activator that binds BAX with high affinity and specificity to the N-terminal activation site and induces conformational changes to BAX, leading to BAX-mediated apoptosis[84]. The histone deacetylase SAHA and its derivatives also have an affinity for BAX and induce its activation [Table 2] but have not been validated in AML models[85]. Other preclinical studies suggested a mitochondrial membrane-mediated spontaneous model of BAX activation. In this model, MOM plays a big role in orchestrating the turnover between cytosolic and membrane-bound BAX, its interaction through the α9 helix and the formation of macropore into the membrane. It is likely that lipids such as cardiolipin play a crucial role in this model[86]. Voltage Dependent Anion Channels

Table 2. Drugs targeting main regulated cell death mechanisms in acute myeloid leukemia

Therapeutic class	Mechanism of action	Drug	Trademark	Phase of development	References
BH3-mimetics	BCL2 inhibition by small molecule	BGB-11417	-	Phase 1 (NCT04771130)	[57]
		S65487	-	Phase 1 (NCT03755154, NCT04742101)	[58]
		APG-2575	Lisaftoclax	Phase 1 (NCT03537482)	[59]
		LP-108	-	Phase 1 (NCT04139434)	[60]
	Direct MCL1 inhibition by small molecule	S63845	-	Phase 1 (NCT02979366, NCT03672695, NCT04629443)	[61]
		AZD5991	-	Phase 1 (NCT03218683)	[62]
		AMG176	Tapotoclax	Phase 1 (NCT02675452)	[63]
		AMG397	Murizatoclax	Phase 1 (NCT03465540)	[64]
CDK9 kinase inhibitor	Indirect MCL1 inhibition by small molecule	Flavopiridol, HMR-1275	Alvocidib	Phase 1 (NCT00407966, NCT03298984), phase 2 (NCT01349972)	[66,69]
		SCH-727965	Dinaciclib	Preclinical	[70,71]
		P 1446A 05	Voruciclib	Phase 1 (NCT03547115)	[72,73]
		AZD4573	-	Phase 1 (NCT03263637)	[74]
		CYC065	Fadraciclib	Phase 1 (NCT04017546)	[75,77]
		TG02-101	-	Preclinical	[78]
BH3 mimetics	BCL-XL inhibition	ABT-263	Navitoclax	Phase 1 (NCT05222984)	-
BH3 mimetics	BFL1 inhibition	-	-	Preclinical	[82,83]
BAX/BAK activator	Direct BAX activation	BTSA1	-	Preclinical	[84]
		WEHI-9625	-	Preclinical	[87,88]
	Mitochondrial iron depletion	AM5	Ironomycin	Preclinical	[53]
TRAIL agonist	Death Receptor 5 (DR5) antibody	IGM-8444	-	Phase 1 (NCT04553692)	[89]
	TRAIL receptor agonist fusion protein	ABBV-621	Eftozanermin alfa	Phase 1 (NCT03082209)	[79,80]
FLIP inhibition	Direct FLIP inhibition	-	-	Preclinical	[92,93]
XIAP inhibition	Antisense oligonucleotide	LY2181308	Gataparsen	Phase 2 (NCT00620321)	[98]
		AEG35156		Phase 1 NCT00363974, phase 2 NCT01018069	[99,100]
	SMAC/DIABLO mimetics	TL32711	Birinapant	Phase 1 NCT01828346 phase 2 NCT01486784, NCT02147873	[102,103]
		ASTX660	Tolinapant	Phase 1 NCT02503423	[105]
		LCL161		Phase 2 (NCT02098161)	[107]
	SMAC/DIABLO mimetics + caspase 8 inhibition	TL32711	Birinapant	Preclinical	[108]
	SMAC/DIABLO mimetics	BV6		Preclinical	[109]
epigenetic therapies	Endogenous retroelements reactivation	-	Epigenetic therapies	Preclinical	[116]
Class 1 FIN	System x_c^- cystine/glutamate antiporter inhibition	-	Sulfasalazine	FDA approved in another indication	[118]
Class 2 FIN	GPX4 inhibition	ML162	Altretamine	FDA-approved in another indication	[122]
Class 3 FIN	GSH metabolism inhibition	APR-246	-	Phase 2 (NCT03931291)	[124]
Class 4 FIN	CoQ oxidoreductase FSP1 inhibition	-	-	Preclinical	[37,38]
Autophagy inhibitors	Lysosomal acidification blockade	-	Hydroxychloroquine	FDA-approved in another indication	[138,139]
	PIK3C3/Vps34 inhibition	SAR405	-	Preclinical	[140,141]
Autophagy inducers	mTOR inhibition	-	Sirolimus	FDA-approved in another indication, phase 1 (NCT01869114)	[143]
		RAD001	Everolimus	FDA-approved in another indication, phase 1/2 (NCT00819546, NCT02638428, NCT01869114)	[144]

(VDACs) are a family of membrane proteins that allow passage of both negatively and positively charged ions, NADH, ATP/ADP and other metabolites across the MOM. In particular, VDAC2 plays a role both in recruiting BAK to the MOM and in inhibiting its activation[17]. WEHI-9625 is a novel small molecule inducing BAK-mediated apoptosis in mice but is completely inactive against human BAK[87,88]. We found that ironomycin sequestered iron into lysosomes and subsequently reduced mitochondrial iron load, promoting the recruitment and non-canonical activation of BAX/BAK in AML *in vitro* and *in vivo* models. Crispr Cas9 screens uncovered the key metabolic and mitochondrial factors regulating this modality of non-canonical RCD[53].

Agents targeting extrinsic pathway

TRAIL Agonism

Agonists of the TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4/5) have been tested in AML, but response rates are low[13]. Novel antibodies against TRAILR1 and TRAILR2 have shown promising preclinical data along with synergy with VEN and are currently tested in phase 1 [Table 2][89]. Eftozanermin alfa (ABBV-621), a second-generation TRAIL receptor agonist binding to the death-inducing DR4 and DR5 receptors, is currently being tested in solid tumors and hematological malignancies [Table 2][90,91].

FLIP inhibition

FLIP (Fas-associated death domain (FADD)-like IL-1β-converting enzyme-inhibitory protein) is a multifunctional protein that plays a role in regulating the death-inducing signaling complex (DISC) and caspase 8 activation. CDK9 inhibitors and bromodomain inhibitors such as JQ1 have been shown to effectively decrease FLIP expression, leading to enhanced sensitivity to TRAIL-induced cell death in cancer[92]. Second-generation FLIP inhibitors have shown preclinical activity in multiple cancer cell lines including AML, and have a high potential for synergy with other apoptosis-targeting agents [Table 2][93,94].

XIAP inhibition

IAPs act as antiapoptotic proteins by inhibiting caspases and are promising therapeutic targets in AML. Inhibition of XIAP has been shown to sensitize AML cells to chemotherapy or BCL2 inhibitors[95,96]. Inhibition of XIAP by antisense strategies or peptides that bind and inhibit the BIR3 domain of XIAP has been tested in phase 1 studies[97-99] but failed in phase 2[100]. The SMAC/DIABLO mimetics (SM) birinapant is one of the most clinically advanced SM and is currently being tested in clinical trials for the treatment of certain solid and hematological cancers[101]. Birinapant showed limited efficacy as a single agent[102]. In a phase 2 randomized, double-blind study, birinapant plus AZA was not superior to AZA alone in advanced myelodysplastic syndromes (MDS) or chronic myelomonocytic leukemias[103]. The drug is also being tested in combination with chemotherapeutic agents and immune checkpoint inhibitors. Preclinical data also indicate that combination of Birinapant plus the multidrug resistance receptor 1 (MDR1) may circumvent birinapant resistance in AML[104]. ASTX660/Tolinapant is a dual antagonist of XIAP and cIAP is currently under investigation in phase 1/2 studies in solid tumors and in combination with hypomethylating agents in AML[105,106]. LCL161, an oral SMAC mimetic, has been tested in patients with myelofibrosis and showed a 30% objective response rate in a recently published phase 2 trial [Table 2][107].

Necroptosis inducers

SMAC mimetics combined with caspase 8 inhibition have been shown to trigger necroptosis in AML preclinical models[108-110]. RIPK1 inhibition enhanced the therapeutic efficacy of the HDAC inhibitor chidamide in FLT3-ITD positive AML, both *in vitro* and *in vivo*[111,112] and increased the efficacy of differentiating agents[113]. Other SMAC mimetics in combination with cytarabine or HMA also showed interesting preclinical results[114]. Therapeutic opportunities for cancer cell death induction through endogenous retroelements (EREs) reactivation have been recently described[115]. EREs are transcriptionally silent within mammalian genomes due to epigenetic mechanisms. Anticancer therapies targeting the epigenetic machinery reinduces ERE expression, inducing antiviral responses associated with consistent increased phosphorylation of RIPK1/3 and MLKL kinases associated with features of necroptosis in treated tumor cells [Table 2][116].

Ferroptosis inducers

Ferroptosis inducers (FINs) belong to four classes[117]. Class I FINs include pharmacological agents that limit intracellular glutathione (GSH) through the inhibition of the system x_c^- cystine/glutamate antiporter, as has been shown in non-leukemic models[28]. The main molecules of this class, which are active in AML models, are erastin and sulfasalazine[118,119]. Class II FINs directly inhibit the detoxifying enzyme GPX4. The lead compound in this class is RSL3 which covalently binds to GPX4. The antitumoral effect of GPX4 disruption has been shown in various models, including AML[120,121]. The FDA-approved alkylating agent altretamine has been shown to directly inhibit GPX4[122]. Class III FINs target GSH synthesis or cysteine synthetase such as buthionine sulfoximine (BSO), an irreversible inhibitor of rate-limiting enzyme in GSH synthesis, and also cisplatin[123]. Focusing on AML, APR-246, a drug known to restore the wild-type conformation of mutant TP53 protein, was shown to actually target GSH metabolism[124,125]. Class IV FINs disrupt the balance of iron metabolism and cellular reactive oxygen species (ROS). Dihydroartemisinin (DHA) belongs to this class[126-128], as well as drugs interfering with the antioxidant system CoQ10[37,38] or drugs modulating PUFA metabolism as they are highly sensitive to lipid oxidation [Table 2][129,130].

Iron chelation and overload play a crucial role in MDS and AML. Therapeutic interventions that modulate iron content and balance within blast cells are at least partially inducing ferroptosis[131]. Our group described in detail the mechanism of action of ironomycin that specifically sequesters ferrous iron into the lysosomes, and induces lipid peroxidation and cell death in several models of cancer stem cells as well as in AML through mitochondrial metabolism disruption[53,54,132].

Agent regulating autophagy

Autophagy deregulation in AML is well documented, but its impact on leukemogenesis remains unclear[133,134]. For example, in AML harboring an FLT3 internal tandem mutation (ITD), mTORC1 activation downstream the RET receptor tyrosine kinase suppresses FLT3 protein autophagic degradation[135]. In contrast, blocking autophagy in FLT3-ITD AML can increase the survival of mice with FLT3-ITD-driven AML[136]. These ambiguous results indicate the complexity of therapeutic intervention based on autophagy in AML. Another layer of complexity lies in the fact that autophagy is a dynamic process. For instance, blocking autophagy cargo can be done at earlier phases (autophagosome biogenesis) or at the later steps (endosome-lysosome fusion).

Autophagy inhibitors

Autophagy inhibition can be achieved by blocking the LC3 interacting regions (LIR) that orchestrate diverse stages of autophagy. This strategy was shown to sensitize cytotoxicity to cytarabine[137]. Late-stage autophagy inhibitors hydroxychloroquine and/or bafilomycin A1 (BafA1) block lysosomal acidification and are active on leukemia blasts and also sensitize cells to chemotherapy[138]. A randomized phase 2 study was recently

published testing the addition of hydroxychloroquine to Imatinib in chronic myeloid leukemia and found no significant differences between the two arms[139]. SAR405, a highly potent small-molecule inhibitor of the phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3)/Vps34, induces a blockage at the late endosome-lysosome step autophagy flux and shows interesting preclinical efficacy in FLT3-ITD AML [Table 1][140,141].

Autophagy inducers

The mTORC1/S6K1 pathway is critical for the regulation of autophagy in AML initiation and progression, as reviewed by Ghosh & Kapur[142]. Therefore, the most studied drugs are the mTORC1 inhibitor sirolimus and everolimus. Despite promising data in preclinical models, clinical studies are ambiguous. Sirolimus combined with the chemotherapy regimen showed a high response rate in patients with baseline mTOR activation[143]. In a phase 1b study from the GOELAMS, everolimus plus chemotherapy improved the clinical outcomes of patients with AML[144]. But mTOR inhibition plus conventional chemotherapy did not show a clinical benefit in two other studies[145,146]. Therefore, mTOR inhibitors still need to be evaluated in AML, and several clinical trials are ongoing (NCT00819546, NCT02638428, NCT01869114).

Pharmacological induction of other cell death pathways

Small-molecule inhibitors of the serine dipeptidases DPP8 and DPP9 (DPP8/9) have been shown to induce pyroptosis in human myeloid cells, including cell lines, primary AML samples and mice models of human leukemia[147]. Constitutive innate immune activation is a pathogenetic driver of ineffective hematopoiesis and MDS, in particular through the NLRP3 inflammasome[148,149] Recent studies found that the inflammasome can be induced by gene mutations involving mRNA splicing by induction of cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING)[150]. As a consequence, the use of immunotherapies targeting inflammatory responses is the subject of many preclinical and clinical studies[151]. Mitotic catastrophe is induced by pharmacological agents targeting the mitotic machinery. Cell cycle inhibitors are currently in development in various hematological diseases, including AML[152,153].

UPCOMING CHALLENGES FOR TARGETING RCD IN AML

The key objectives of translating preclinical results into the clinic are to identify drugs that will treat resistant cells by inducing RCD, detect the population of patients who will respond to a given RCD and design new RCD inducers with high efficacy and low toxicity.

Target resistances with RCD

Cells resistant to treatment, called "persister cancer cells", are largely responsible for relapse[154]. These cells develop antiapoptotic mechanisms as well as altered metabolism rendering them more susceptible to alternative RCD, in particular autophagy or ferroptosis that are directly related to the metabolic state of the cells[155,156]. For instance, preclinical data showed the x_c^- inhibitory activity of salazopyrine and its efficacy against primary AML samples in *ex vivo* cultures and in patient-derived AML models[118]. These preliminary results led to the hypothesis that the drug could be repositioned in AML. A clinical trial testing the addition of salazopyrine to ICT in older AML patients, named SALMA (EUDRACT no: 2022-001269-11), will be enrolling soon. Another example is the resistance to VEN, which is mediated through various mechanisms, including BCL2 family protein expression and occupation (MCL1 dependency), cellular differentiation state (monocytic versus stem cell-like), cellular metabolic state or sensitivity to mitochondrial machinery disruption[11]. One of the major limitations that emerged from both *in vitro* and clinical studies with the BH3-mimetics is the low sensitivity of TP53 mutated blasts to this class[157-159]. Strategies of treatment that overcome TP53-dependent apoptosis can be used, such as ironomycin that directly activates BAX/BAK in a BCL2-family protein-independent manner[53]. Optimized clinical-grade derivatives of ironomycin with better therapeutic windows are currently under development[160].

Identify new biomarkers

The second challenge is to identify the population of patients who will benefit from RCD inducers. Recent findings showed that ferroptosis-associated gene signatures can be assessed by transcriptomic methods such as RNA sequencing. These signatures predict survival and could possibly guide therapeutics by selecting patients who could be treated by the use of ferroptosis inducers[161-164]. Necroptosis transcriptomic signatures have also been found in MDS, indicating that such predictors could identify patients who would benefit from necroptosis inducers[165]. Functional assays such as BH3-profiling have been shown to be a highly efficient companion test that can predict response to the BH3-mimetics inhibiting BCL2 family proteins[20,166]. Our team published a translational proof-of-concept study in which relapsed or refractory AML patients were selected according to molecular and *ex vivo* drug sensitivity profiles[167]. Future clinical trials using companion tests based on molecular or functional approaches will confirm the feasibility and efficacy of this strategy.

Discovery of new RCD inducers

The use of targeted therapies has transformed AML treatment. Small molecule inhibitors of genes that underwent common somatic mutations, such as FLT3 or IDH inhibitors, have been approved recently for AML relapsed or refractory patients[168]. However, most of the patients cannot benefit from targeted therapies, because of a lack of targetable genetic alterations. On the contrary, BCL2 inhibition success story is based on the AML blast dependency to apoptosis independently of AML targetable mutations. Recent findings showed that AML cells are sensitive to ferroptosis induction, suggesting that efforts should be made to develop new ferroptosis inducers[169]. A validated approach is to use an unbiased strategy by performing high throughput screening of chemical libraries for their ability to induce ferroptosis (or other RCD) *in vitro* and *in vivo*[122]. In parallel, a better understanding of the ferroptotic molecular pathways can be obtained using CRISPR Cas-9 resistance screens of a given ferroptosis inducer that will identify crucial genes involved in cell death. We recently used this strategy to uncover the molecular mechanism of ironomycin[53]. This integrative strategy will help to design new tailored drugs and pave the way for future clinical trials based on RCD.

CONCLUSION

Treatment of AML has remained unchanged for more than 40 years with a one-size-fits-all intensive chemotherapy approach for eligible patients. In 2017, the FDA approved the utilization of two targeted therapies for patients with a molecular alteration in FLT3 or IDH genes[168]. Another significant advancement was made in 2020 with the publication of the VIALE-A study reporting the efficacy of VEN for the treatment of AML patients who are not eligible for intensive treatment[10]. Despite higher response and survival rates than before, there is still improvement to be made in the management of AML patients. A better understanding of molecular RCD pathways in collaboration with integrative translational studies will allow the design of new drugs or the repositioning of older ones to overcome resistance in AML.

Intrinsic apoptosis is initiated by the formation of pores permeabilizing the outer membrane of mitochondria (MOMP) induced by the oligomerization of BAX recruited from the cytosol and BAK inserted at the mitochondrial membrane. BAX and BAK interact with a series of antiapoptotic inhibitors proteins (BCL2, BCLXL, MCL1) and proapoptotic activator proteins (BIM, PUMA, NOXA, BID). MOMP induces a release of cytochrome c that cleaves caspase 9 and subsequently activates the effector caspases 3 and 7, leading to cell death. Extrinsic apoptosis is triggered by the ligation of cell death receptors (FAS, TRAIL and TNFR), which allow the assembly of the "death-inducing signaling complex" (DISC) and the

subsequent activation of caspase 8. Caspase 8 induces the cleavage of BID, leading to the activation of BAX and intrinsic apoptosis and the direct activation of caspases 3 and 7, finally leading to cell death. Inhibitors of apoptosis (IAP) family, including XIAP, cIAP1 and cIAP2, are inhibitors of caspase 9 and the death receptor TNFR, whereas FLIP is the inhibitor of caspase 8. SMAC/DIABLO is a major inhibitor of IAPs. Necroptosis is induced by the ligation of cell death receptors and the activation of the receptor-interacting protein RIPK1 and RIPK3 that interacts with protein complex mixed lineage kinase domain-like pseudokinase (MLKL). Phosphorylation of MLKL results in the formation of pores on the cell membrane, followed by cell death. IAPs and Caspase 8 inhibit RIPK1 activation and block necroptosis. Ferroptosis is the result of lipid peroxidation triggered by the direct inhibition of the antioxidant enzyme GPX4 or by the blockage of the x_c^- cystine/glutamate antiporter (xCT-). Depletion of cysteine import and intracellular glutathione (GSH) increases lipid peroxides which is exacerbated by the Fenton reaction, where H_2O_2 and iron react to generate hydroxyl radicals. Autophagy is a process in which cytoplasmic components, including intracellular organelles, are degraded by the lysosomes. The first step of autophagy is the formation of phagophores, followed by the generation of double-membrane autophagosomes regulated by autophagy-regulated genes (ATG) and lysosomal fusion. Autophagy blockage or excess can induce cell death.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the review: Garciaz S, Miller T, Collette Y, Vey N

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Cancer Drug Resistance

Review

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Anti-BCMA novel therapies for multiple myeloma

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Abstract

Recent advances in multiple myeloma therapy have increased the depth of response and ultimately survivals; however, the prognosis remains poor. The BCMA antigen is highly expressed in myeloma cells, thus representing a target for novel therapies. Several agents that target BCMA through different mechanisms, including bispecific T cell engagers drug conjugated to antibody and CAR-T cells, are now available or under development. Immunotherapies targeting BCMA have shown good results in efficacy and safety in multiple myeloma patients previously treated with several lines of therapy. This review will discuss the recent development of anti-BCMA targeted treatments in myeloma, with a special focus on currently available agents.

Keywords: Multiple myeloma, BCMA, belantamab, teclistamab, CART

INTRODUCTION

Multiple myeloma (MM) is a clonal plasma cell (PC) disorder accounting for 10% of hematologic neoplasms[1]. Novel therapies such as proteasome inhibitors (PI), immunomodulatory drugs (IMiDs), and anti-CD38 monoclonal antibodies (mAbs), together with autologous stem cell transplant (ASCT), have significantly improved treatment outcomes of newly diagnosed MM patients with a continuous increase of the overall survival (OS) that today reaches a median of 10 years[2-9]. However, MM patients still do relapse

and MM is considered an incurable disease[10,11]. In particular, triple-class refractory (refractory to PI, IMiDs, and anti-CD38 antibody) and penta-refractory (first and second-generation PIs, two generations of IMiDs, anti-CD38 antibody) patients have a median OS of 5.6 months, especially in the presence of high-risk cytogenetics (HR)[12-20] or positive minimal residual disease[21-26]. Therefore, novel therapies, especially for relapsed/refractory myeloma patients (RRMM), are necessary[25-26]. BCMA is a B-cell maturation antigen highly expressed in myeloma cells, thus offering an encouraging potential target for novel treatments[27-28].

BCMA in multiple myeloma

BCMA, i.e., CD269 or TNFRSF17, is a TNF receptor superfamily 17 member, expressed on differentiated plasma cells and plasmablasts under physiological conditions and nearly on all MM tumor cells[29-31]. BCMA ligands include APRIL (A Proliferations-Inducing Ligand) and BAFF (B-cell activating factor) which are involved in the maturation and differentiation of PCs[32]. APRIL can bind to BCMA more avidly than to BAFF, and both can induce BCMA downstream signals to PI3K-PKB/Akt (i.e., phosphoinositide-3-kinase-protein kinase B/Akt), to RAS/MAPK (i.e., rat sarcoma/mitogen-activated protein kinase), and also to NF-κB (i.e., nuclear factor kappa-B), inducing increased plasma cells proliferation and survival[31,33-37]. Interestingly, PCs long-term survival in BCMA$^{-/-}$ mice is defective, suggesting BCMA is crucial for a sustained humoral immune response[38-39].

BCMA is overexpressed in myeloma PCs compared to normal ones, and its expression levels are elevated regardless of the stage of MGUS (monoclonal gammopathy of undetermined significance), SMM (smoldering multiple myeloma), and symptomatic MM[40-41]. Moreover, compared to healthy controls, APRIL and BAFF serum levels are 5-fold higher in myeloma patients. Recent studies showed that osteoclasts could be stimulated to produce more APRIL by MM cells, thus producing an immunosuppressive microenvironment[31,35,42] Interestingly, MM cell proliferation can be reduced, in a mouse xenograft model, by a moAb directed against APRIL. Anti-BCMA immunotherapies, together with APRIL inhibition, can defeat MM-induced immunosuppressive microenvironment and intensify the ADCC (antibody-dependent cell-mediated cytotoxicity) against myeloma cells[31,35,43].

sBCMA is the soluble form of BCMA, and it is produced by a γ-secretase acting on membrane BCMA[44]. sBCMA levels have been related to plasma cell infiltration in the bone marrow and may predict MM patients' outcome. Indeed, some studies have shown that after MM treatment, the responsive patients resulted in lower sBCMA levels compared to patients with progressive disease[45-47]. Moreover, MGUS and SMM patients with higher levels of sBCMA showed a higher risk of progression to MM[48-49]. Thus, sBCMA might be used as a biomarker for disease progression and treatment response, allowing appropriate therapeutic management in case of drug resistance[10,50]. Additionally, one preliminary study in patients with non-secretory myeloma, for whom bone marrow aspirate and PET-CT scan are the only methods for disease monitoring, has shown that sBMCA levels correlate with the bone marrow PC infiltration, although this need to be confirmed[45-46]. Further studies are needed to validate sBCMA as a novel biomarker for MM and no approved diagnostic tool for measuring serum levels of sBCMA is available yet[10].

Finally, sBCMA reduces BCMA expression on PCs' surface, thus resulting in reduced efficacy of BCMA-targeted therapies and MM cells' immune escape[27]. Additionally, authors showed that sBCMA at high levels might interfere with anti-BCMA therapy, thus reducing effective binding to MM cells[51]. Preclinical studies of γ-secretase inhibitor (GSI) have shown that it may decrease sBCMA levels and increase MM cells expressing surface BCMA, thereby improving response to BCMA chimeric antigen receptor T cell (CAR-T) therapy. Hence, the association of a GSI and BCMA-targeting therapy in MM patients is being evaluated in early-phase clinical trials[2,52].

BCMA-TARGETED TREATMENT IN MULTIPLE MYELOMA

The evidence that BCMA could be a suitable target for effective antitumor activity in preclinical studies led to the development of drugs targeting BCMA with several mechanisms [Figure 1]. Presently, BCMA-targeted therapies available are represented by: antibody-drug conjugates (ADCs), bispecific T cell engager (BiTEs), and chimeric antigen receptor (CAR)-T cells [Table 1][53,54].

BCMA antibody drug conjugates

Antibody-drug conjugate (ADC) consists of a monoclonal antibody directed against a tumor- antigen and a cytotoxic agent inducing cell death (payload). ADC is internalized after binding to the related antigen on the tumor cell's surface, then the linker is hydrolyzed inside of the lysosomes or endosomes and the payloads are released to cause cell death. ADCs can selectively target malignant cells with great efficiency on tumor cells and limited toxicities. Auristatin is a tubulin polymerase inhibitor used as a payload for MM[55-60].

Belantamab Mafodotin (GSK2857916)

Belantamab mafodotin (Bel) is a humanized IgG1 ADC, first-in-class, originally approved by the FDA (US Food and Drug Administration) as monotherapy in relapsed myeloma patients treated with four prior therapies including a proteasome inhibitor, anti-CD38 monoclonal antibody, and an immunomodulatory agent[61]. Bel is formed by an antibody directed to BCMA and covalently linked to MMAF (the microtubule inhibitor monomethyl auristatin F)[62]. After binding to BCMA on MM plasma cell, Bel is internalized and MMAF is released, provoking cell-cycle arrest and apoptosis[63]. Other effects that seem to be mediated by Bel-binding BCMA are ADCC and antibody-dependent cellular phagocytosis (ADCP)[64,65].

The multicenter phase I trial (DREAMM1) enrolled 73 RRMM patients. An ORR of 60% and PFS of 12 months were reported with acceptable toxicities. Corneal toxicity resulted in the most common non-hematologic side effect[66,67]. Subsequently, the phase II registrational study DREAMM2 enrolled 196 MM patients. The recommended dose was intravenous 2.5 mg/kg, Q3W. Reported ORR was 31%, with toxicities confirmed as manageable. A program was established to evaluate possible Keratopathy (Risk Evaluation and Mitigation Strategy, REMS) prior to drug administration[68-71]. Bel is currently being studied in different combination regimens in MM patients. The randomized, phase II study DREAMM4 is investigating Bel with pembrolizumab in patients with MM refractory to multiple lines of therapy. The DREAMM5 is testing Bel with other mAbs, such as isatuximab. The DREAMM-6 trial is exploring the combination of Bel, bortezomib, and dexamethasone *vs.* Bel, lenalidomide, and dexamethasone, while the DREAMM-7 and the DREAMM-8 studies are comparing Bel, bortezomib and dexamethasone *vs.* daratumumab, bortezomib and dexamethasone and Bel, pomalidomide and dexamethasone *vs.* pomalidomide, bortezomib and dexamethasone, respectively. Finally, the DREAMM-9 is testing Bel in the induction therapy in NDMM patients[2,10,72,73]. However, in November 2022, the FDA withdrew belantamab's US marketing authorization as the DREAMM-3 trial (Bel *vs.* pomalidomide in combination with low-dose dexamethasone in RRMM) did not meet its primary endpoint of PFS (11.2 *vs.* 7 months, HR 1.03; 95%CI: 0.72-1.47). Sustainability could be a reason. Other Bel studies are ongoing, and results are awaited. Other studies including different anti-BCMA mAbs as well as ADCs targeting BCMA are ongoing[74-76].

BCMA bispecifics

BITEs are bispecific T cell engagers and represent a different modality of immunotherapy targeting BCMA. These agents are engineered proteins with two different antigen-binding fragments that bind to MM cells and T cells, thus creating an immunological synapse with direct plasma cell killing by T-cells[77-79]. The two common antigens involved are CD3 and CD16, and BCMA is the target of MM plasma cells. Many studies

Table 1. Characteristics of currently approved BCMA targeted agents

Drug	Mechanism of action	Regimen of administration	Adverse effects	ORR/CR (%)*	PFS (months)*
Belantamab (ADCs)	Monoclonal antibody conjugated with a cytotoxic agent	Intravenous (every 21 days)	Corneal toxicities Thrombocytopenia	31/3	2.8
Teclistamab (BITEs)	Fully humanized IgG4 bispecific antibody redirecting, CD3-positive T-cells to BCMA	Subcutaneous (weekly)	CRS ICANS Hematological toxicities	63/39	11.3
Idecabtagene Vicleucel (CAR-T)	BCMA targeted CAR-T incorporating anti-BCMA antibody costimulation domain, CD3ζ signaling domain	Single intravenous infusion	CRS ICANS Hematological toxicities	73/33	8.8
Ciltacabtagene Autoleucel (CAR-T)	BCMA-targeted CAR-T-cell product with two single anti-BCMA domain antibodies, CD3-ζ signaling domain costimulatory domain	Single intravenous infusion	CRS ICANS Hematological toxicities	97/67	Not reached

*Data from the registrational study; CR: complete response; ORR: overall response rate; PFS: progression-free survival.

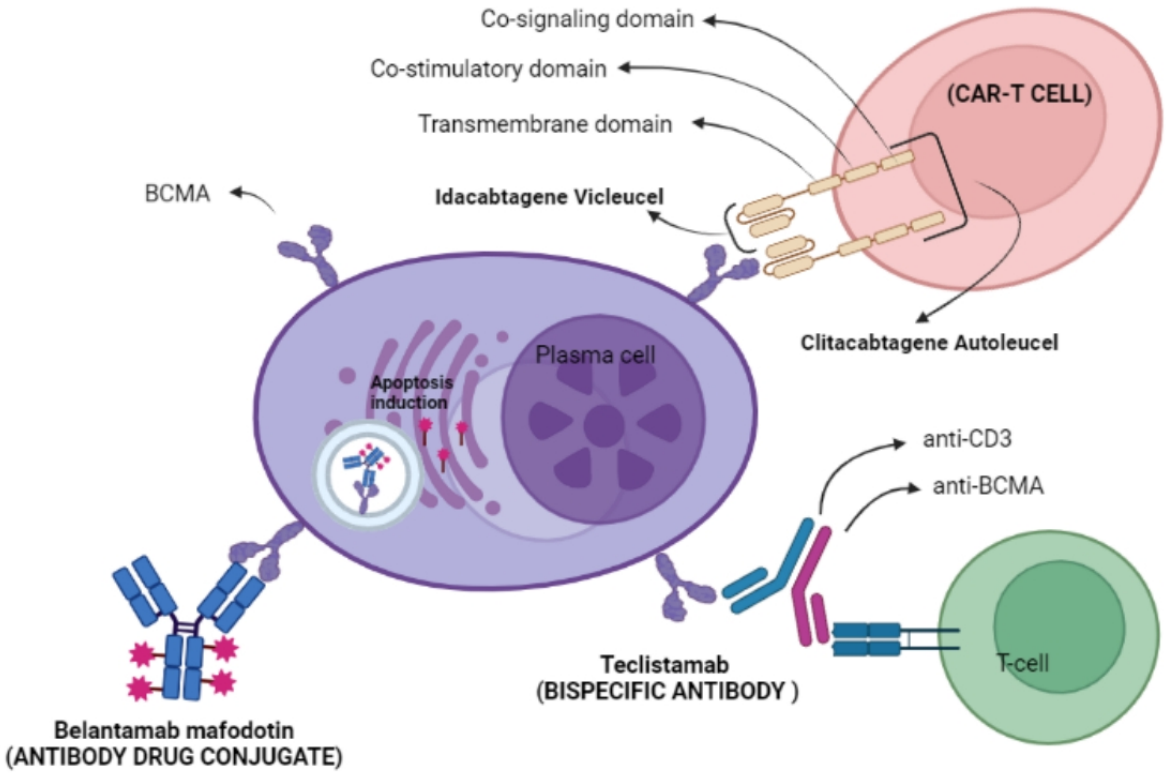

Figure 1. CAR: Chimeric antigen receptor.

with BITEs utilizing BCMA showed great efficacy with moderate toxicity, such as CRS (cytokine release syndrome) and associated neurotoxicity syndrome (ICANS)[80-82].

Teclistamab (JNJ-64007957)

In the MajesTEC-1 clinical trial, Teclistamab (Tec) was tested as an IgG4 bispecific antibody targeting CD3 on T-cells and BCMA in RRMM. Included patients were heavily pretreated, with two-thirds of them triple-class refractory and 30% penta-refractory. Tec was initially administered intravenously or subcutaneously in different cohorts, and safety was particularly improved, particularly in terms of reduced CRS, for the latter formulation. The recommended dose was 1500 μg/kg weekly subcutaneously, after two escalating doses of 60 and 300 μg/kg. The ORR was 63% (median PFS 11.3 months). CRS was observed in 72% of the patients (only 1 patient with a grade 3) and Il-6 inhibitor tocilizumab was needed in 37% of patients. The most common neurotoxicity reported was headache in 8% of the patients[83-86]. Those results were followed by Tec authorization for marketing as monotherapy in MM patients who showed disease progression during the last of three prior therapies, including a proteasome inhibitor, an immunomodulatory agent, and an anti-CD38 antibody[87].

Others BITEs currently studied are Elranatamab (PF-06863135), ABBV-383, and alnuctamab (CC-93269). In addition, novel tri-specific agents that target BCMA are under preclinical evaluation and are demonstrating high clinical potential[88-97].

BCMA CAR-Ts

CART (Chimeric antigen receptor T) cell therapy act as cell-mediated immunotherapy. Briefly, after an in vitro gene transfer strategy, the patient's T cells acquire the ability to can recognize tumor antigens (mostly used is BCMA) on MM plasma cells and thus destroy them. The CARs are formed by a receptor with an extracellular portion that binds to the antigen and an intracellular signaling domain. Moreover, the extracellular portion is formed by a single-chain variable fragment, i.e., scFv, connected to a transmembrane domain. CD28 is used as a costimulatory molecule. The final product results in a combination effect of mAbs and T cells cytotoxicit[98-103]. Leukapheresis of the patient's T cells is the first step of CART generation. Thereafter, the scFv and costimulatory domains are introduced with a viral vector. Before reinfusion, patients usually receive a conditioning regimen of fludarabine and cyclophosphamide (a chemotherapy regimen used to achieve lymphodepletion) to decrease autologous T cells and permit CARTs proliferation[104,105].

BCMA represents an ideal target for CAR-T therapy, and to date, two autologous BCMA-targeting CAR-Ts have been approved by the FDA, but several are being investigated in clinical trials[106-108]. BCMA is also being tested combined with CD19 for CAR-Ts multiple targeting[109-111]. A good efficacy has been demonstrated in early-phase clinical trials with bispecific CAR-Ts that target BCMA, CD19, or CD38[112]. Future alternative approaches could be represented by allogenic BCMA CAR-T cells or CAR-NK (CAR-natural killer), which are now investigated in early clinical trials[113-121].

Ide-Cel, idecabtagene vicleucel (bb2121)

Ide-Cel is a CAR-T of the second generation that targets BCMA. Ide-Cel includes a CD3ζ signaling domain and an scFv, a costimulating domain. A great efficacy has been shown in preclinical experiments against MM plasma cells. It is independent of levels of BCMA expression or sBCMA levels[107]. Ide-Cel showed an ORR of 85% and a median PFS of 11.8 months in heavily pretreated MM patients in a phase I study. Toxicities such as CRS and ICANS (mostly grade 1-2) were observed in 76% and 42% of patients, respectively[122]. The KarMMa phase II study was conducted in 128 MM patients who had previously received three or more lines of therapy, including a PI, an IMiD, and an anti-CD38 mAb. CAR-Ts infusion produced an ORR of 73%. Also, MRD negativity at 10^{-5} was seen in 26% of the patients (median PFS 8.8 months, 20.2 months when CR was achieved). Of note, when CAR-T was employed in high-risk disease

patients (i.e., penta-refractory disease, extramedullary disease, or high-risk cytogenetic), results were confirmed. Toxicities were acceptable (CRS 84%, but only 7 patients (5%) with ≥ grade 3; ICANS 18%, with ≥ grade 3 in 4 (3%) MM patients[123]. Ide-Cel was approved by the US FDA thereafter for MM patients treated with four lines of therapy (comprehensive of a PI, an IMiD, and an anti-CD38 mAb)[124]. Ide-Cel is now being used in several trials to explore its efficacy in various scenario, including the use in first-line therapy or at early relapse[125-127].

Cilta-cel ciltacabtagene autoleucel LCAR-B38M/JNJ-4528; Carvykti

Cilta-cel is a CAR-T-cell targeting BCMA with two antibodies to increase the binding avidity, a CD3-ζ signaling domain and a 4-1BB costimulatory domain[109]. In a recent phase I clinical trial, responses were high (ORR 88%) in RRMM patients after three or more prior lines of therapy (median PFS of 15 months). Toxicities were mostly grade 1-2 (CRS 90%, ICANS in 1 case)[128]. Subsequently, Cilta-cel was tested in 97 MM patients previously treated with multiple lines of therapy, with 40% of them being penta-refractory (CARTITUDE-1 trial). Interestingly, the response rate was quite high (> VGPR in 95%, MRD undetectable at 10^{-5} was achieved in 92%). Reported CRS and ICANS were similar to the previous study, but hematologic toxicities occurred more frequently (grade 3-4)[129]. Cilta-cel was then approved by FDA, in February 2022, for RRMM patients treated with > 4 prior lines of therapy including an anti-CD38 mAb, an IMiD, and a PI[130]. Recent ongoing phase III trials are CARTITUDE-2, evaluating cilta-cel efficacy and safety in different clinical settings in RRMM[131]; CARTITUDE-4, comparing Cilta-cel *vs.* pomalidomide, bortezomib and dexamethasone (PVd) *vs.* daratumumab, pomalidomide and dexamethasone (DPd) in RRMM; CARTITUDE-5, comparing bortezomib, lenalidomide, and dexamethasone (VRd) and Cilta-cel *vs.* VRd followed by lenalidomide and dexamethasone (Rd) therapy in transplant-ineligible patients MM at diagnosis[132].

BCMA, DRUG RESISTANCE, AND MM

While the efficacy and safety of BCMA-targeting agents have been demonstrated, data regarding drug resistance are also emerging, though the exact mechanisms of resistance towards these agents have not been fully understood[133]. Bone disease could be a reservoir for disease recurrence and a mechanism of resistance. Imaging is an important tool to detect residual disease outside the bone marrow or in extramedullary disease, although it is not known how BCMA antigen could be expressed on plasma cells outside the bone marrow. PET-CT is the gold standard technique to detect active disease and translated from lymphomas to MM[134,135]. In addition, whole body-MRI studies showed equal sensibility *vs.* PET-CT and can be used[136]. Downregulation of BCMA on PCs surface could be associated with resistance in a similar way as it has been described for CD19 and CD20 target therapies. Multi-targeted immunotherapies or the combination of BCMA targeting agents with γ-secretase inhibitors could overcome BCMA loss and both strategies are under investigation in clinical trials[52].

Humoral and cellular immune responses could limit the persistence of BCMA CAR-T, leading to loss of efficacy and disease relapse. Alternative manufacturing processes, such as the application of human scFVs or the removal of the light-chain domain from the CAR antigen-binding domain, have been demonstrated to reduce CAR-T immunogenicity. In addition, BCMA CAR-T persistency could be increased by the addition of a phosphoinositide 3 kinase inhibitor during ex vivo culture to augment the memory-like T cells of the final product. Besides, allogenic CAR-T could overcome resistance related to T cell exhaustion which may be present in RRMM patients[137-139].

Eventually, the tumor microenvironment is now considered to play a central role in promoting MM cell growth and has also been associated with drug resistance. Combination of BCMA targeting drugs with

immunomodulatory agents could overcome this intrinsic mechanism of resistance, while trials are evaluating the next generation of armored CAR-T cells engineered to secret immunostimulatory cytokines or antibodies against inhibitory immune checkpoint receptors such as PD-1 and PD-L1.

DISCUSSION

Despite novel therapeutic advantages in recent years, MM remains incurable. BCMA immunotherapies are a novel anti-MM therapeutic approach that holds promise to improve MM survival in the future. ADCs, BITEs, and CAR-T cells are the newest therapeutic options targeting BCMA. Early clinical trials showed great efficacy and safety even IN MM patients treated with > 4 prior therapy lines. Since comparative studies of anti-BCMA targeted therapies are still lacking, it is not yet known whether one of these classes of agents is superior to another; however, they all have unique toxicities and logistical challenges. ADC is an interesting and efficacious therapy, but corneal toxicities need further understanding. Bispecific antibodies are therapies that can be used with excellent clinical activity. Disadvantages of bispecific antibodies could be their short lifetime and the need to start treatment in a hospital setting, as severe CRS/ICANS side effects usually appear at the beginning of therapy.

CAR-T cells are also a great option, as clinical trials reported high response rates in heavily pretreated MM patients. The main drawbacks of CAR-T cells include manufacturing time and expenses, leukapheresis necessity, and use of chemotherapy and infusion in a hospital setting for toxicities management. In addition, a relevant mechanism of resistance could be represented by the limited CAR-T growth and contact with the adverse plasma cell myeloma microenvironment, thus resulting in limited therapeutic effects after one year[140]. To overcome these problems, new strategies are currently under investigation utilizing combos of drug agents with CAR-T, maintenance therapies after CAR-T, novel methods to extend CAR-T's duration, and implementing CAR-T production[141]. Additionally, the combination of a checkpoint inhibitor with CAR-Ts is being tested as it may offer an advantage of reducing T cell downfall[142].

The appropriate timing when to utilize a BCMA-targeted therapy is presently under investigation, with trials evaluating its role in earlier lines of therapy, including frontline. In fact, T cell-stimulating agents, such as CAR-T cells and BITEs, could probably produce deeper and longer responses if used at diagnosis or after only one or two lines of therapy, when MM patients are not heavily treated and may be at lower risk for T cell exhaustion.

In conclusion, therapies that target BCMA will play an important role in MM therapy, with the ambitious purpose of improving the cure rate; however, further investigations are still necessary to better define their real impact in clinical practice.

DECLARATIONS

Authors' contributions

Made substantial contributions to the conception and design of the study: Sammartano V, Gozzetti A
Wrote the manuscript: Sammartano V, Franceschini M, Fredducci S, Caroni F, Ciofini S, Pacelli P
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Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Heterotypic signaling of cancer-associated fibroblasts in shaping the cancer cell drug resistance

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Abstract

The context-dependent reciprocal interaction between the cancer cells and surrounding fibroblasts is imperative for regulating malignant potential, metabolic reprogramming, immunosuppression, and ECM deposition. However, recent evidence also suggests that cancer-associated fibroblasts induce chemoresistance in cancer cells to various anticancer regimens. Because of the protumorigenic function of cancer-associated fibroblasts, these stromal cell types have emerged as fascinating therapeutic targets for cancer. However, this notion was recently challenged by studies that targeted cancer-associated fibroblasts and highlighted the underlying heterogeneity by identifying a subset of these cells with tumor-restricting functions. Hence, it is imperative to understand the heterogeneity and heterotypic signaling of cancer-associated fibroblasts to target tumor-promoting signaling processes by sparing tumor-restricting ones. In this review, we discuss the heterogeneity and heterotypic signaling of cancer-associated fibroblasts in shaping drug resistance and also list the cancer-associated fibroblast-targeting therapeutics.

Keywords: Tumor microenvironment, CAFs, heterogeneity, ECM, metabolic reprogramming, heterotypic signaling, drug resistance, natural products

INTRODUCTION

Accumulation of genetic or epigenetic aberration may be important for the transformation of normal epithelial cells but not sufficient to induce malignant potential. Context-dependent interaction between cancer cells and tumor microenvironment components is imperative for malignant progression[1]. Tumor microenvironments consist of various kinds of non-cancerous cells such as fibroblasts, macrophages, mesenchymal stem cells (MSCs), pericytes, endothelial and immune cells, and extracellular matrix (ECM) known as tumor stroma[2]. Fibroblasts constitute a major component of tumor stroma and exhibit multipronged functions in tumor progression[3,4].

Fibroblasts could be considered cockroaches of the human body as they thrive under severe stress and can even be isolated from decaying/dead tissue. Fibroblasts are quiescent cell types and synthetically and metabolically less active[3]. Upon activation, fibroblasts play a critical role in the wound healing process by remodeling ECM as well as secreting various growth factors and chemoattractant cytokines which ultimately regulate epithelial proliferation and immune cell infiltration[3,5,6]. Dysregulation of their activation leads to the formation of scar and fibrotic diseases. Fibroblasts associated with cancer, termed cancer-associated fibroblasts (CAFs), show functional and molecular differences from normal fibroblasts. Fibroblast activation by the secreted factors, cell-matrix or cell-cell contacts with cancer or other stromal cells leads to the CAF phenotype acquisition[2,7-9]. CAFs have been reported to exhibit higher migratory and contraction potentials along with synthesizing and remodeling ECM, reminiscent of myofibroblasts[8,9]. Several reports show that CAFs secrete a myriad of growth factors and cytokines which are critical for several facets of tumor progression. CAFs were known to regulate several hallmarks of cancer by directly influencing cancer cell proliferation, migration, invasion, and angiogenesis[7-9]. Our earlier study also reported that osteopontin (OPN)-activated CAF-derived CXCL12 promotes epithelial-to-mesenchymal transition (EMT) in breast cancer cells[8]. Moreover, CAFs are known to shape the tumor immune microenvironment through the elevated expression of immunosuppressive cytokines and immune checkpoint proteins that results in immunosuppression and tumor progression[10]. More importantly, CAFs are reported to induce drug resistance and cancer relapse in different cancers by different mechanisms including the induction of EMT, activation of stemness pathways, ECM remodeling, and dysregulated metabolism[11]. Due to their important functions in tumor progression, CAFs have emerged as an intriguing therapeutic target for the clinical control of cancer. However, the studies focused on targeting CAFs for the management of cancer have challenged this dogma. Of note, genetic ablation of the CAF population or fibrosis induces immunosuppressive environment in pancreatic ductal adenocarcinoma (PDAC) which in turn promotes EMT and invasion in cancer cells, leading to tumor progression with poor disease outcomes[12]. In addition, targeting the hedgehog (Hh) pathway in CAFs led to more aggressive and poorly differentiated PDAC with reduced stromal content and survival[13,14]. The above report highlights the presence of a subset of CAFs with tumor-restricting functions. Understanding the heterogeneity of CAFs and their heterotypic signaling might help in tailoring therapeutic intervention that selectively targets tumor-promoting CAF population and spares tumor-restraining ones. This review focuses on CAF heterogeneity and heterotypic signaling in regulating drug resistance to cancer therapies. This review also highlights several current CAF-targeted therapies for the treatment of different cancer types.

NORMAL FIBROBLASTS AND ACTIVATED/CANCER-ASSOCIATED FIBROBLASTS

During the generation of the third germ layer or mesoderm, primitive mesenchymal cells (primary mesenchyme) first appear when the epiblast undergoes EMT[15]. Most of the active mesenchymal cells undergo apoptosis after the completion of tissue development, whereas few cells attain a quiescent phenotype, which was first observed by Virchow[16] and eventually named fibroblasts. Normal fibroblasts are elongated cells with extended cell processes that exhibit a fusiform or spindle-like shape. These are generally

present in connected tissues where they are embedded within ECM which consists largely of type I collagen and fibronectin[17]. A specific marker of quiescent fibroblasts is still missing; however, fibroblast-specific protein 1 (FSP1) and vimentin are considered as the closest. Normal fibroblasts also express integrins which are the mediators of the interaction of fibroblasts with their surrounding microenvironment[17]. Additionally, normal fibroblasts are characterized by low metabolic activity and lack of mobility[3].

Fibroblasts can be activated to acquire activated/myofibroblast phenotype, which is associated with enhanced proliferative activity and increased synthesis of ECM proteins such as type I collagen, tenascin C, extra domain A (EDA)-splice variant of fibronectin, and secreted protein acidic and rich in cysteine (SPARC)[17]. Fibroblast activation can be promoted by various stimuli generated from tissue injury or damage, including transforming growth factor beta (TGF-β), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), and interferon-γ (IFNγ), interleukin (IL-6), mechano-transductions and enzymes[17-19]. Upon activation, these cells exhibit prolific protein synthesis and higher contraction potential that is crucial for wound healing and the production of connective tissues[3]. In physiological conditions, myofibroblasts play a critical role in wound healing and repairing damaged tissues[19-22]. Upon the completion of their function, these cells are cleared by programmed cell death, apoptosis[23]. However, if the injury is perpetual or dysregulation of the cell death program of these cells, it can lead to hyperproliferation and accumulation of myofibroblasts which culminates in a condition known as fibrosis[24-26].

"Tumors are depicted as wounds that do not heal" as they undergo continuous stromal remodeling and vascular growth, reminiscent of the wound repair program. Similar to wound healing process, activated fibroblasts/myofibroblasts are also present in tumors and are known as CAFs[9]. A diverse set of tumor or stroma-derived factors, including TGF-β1, OPN, and IL-1β, drive the transition of resting fibroblasts to CAFs by regulating Akt, ERK, MAPK, SMAD and NF-κB signaling pathways[8,27-29]. In an activation state, CAFs attain increased contractibility features and migratory potentials, which enables the CAFs to remodel ECM and aid in reciprocal interaction with cancer cells[3,30,31]. Different CAF-specific markers were identified to characterize activated CAFs, such as alpha-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), FSP1 (also known as S100A4), Integrin β1 (CD29), platelet-derived growth factor receptor α or β (PDGFRα/β) or podoplanin (PDPN)[32]. PDGFRs are a class of RTKs, known to be involved in tumor-fibroblast interactions[33]. In contrast to wound healing, but similar to organ fibrosis, the fibroblasts at the tumor site remain perpetually activated and form fibrous growth in the tumor, referred to as desmoplastic reaction/stroma[34]. Moreover, it was observed that senescent fibroblasts, which resemble myofibroblasts, also support preneoplastic tumor growth via secretion of OPN[35,36].

ORIGIN OF CAFS

The expression of different kinds of markers in CAFs indicates the heterogeneous generation and different cellular sources of these cells. CAFs can be originated from epithelial cells through the EMT [Figure 1]. According to a report, epithelial cells undergo specialized EMT by MMP-driven oxidative stress-associated DNA oxidation and mutations that lead to transdifferentiation of these cells into activated myofibroblasts[17,37]. This hypothesis is mainly supported by genetic studies conducted on breast cancers. These studies have reported somatic mutations in TP53 and phosphatase and tensin homolog (PTEN), and gene copy number alteration at other loci in stromal CAFs, similar to mutations in epithelial cells. Moreover, p53 inactivation in stromal fibroblasts and genetic inactivation of PTEN in CAFs promote tumor progression in breast carcinoma models[38-40]. Collectively, these data indicate that the tumor-promoting activity of CAFs may depend on somatic mutations in these tumor suppressor genes. In addition, somatic alterations were frequently detected (> 30%) in tumor cell-surrounding fibroblasts[39,40]. Similarly, CAFs might be generated from cancerous epithelial cells by EMT [Figure 1][41]. The EMT renders cancer cells to

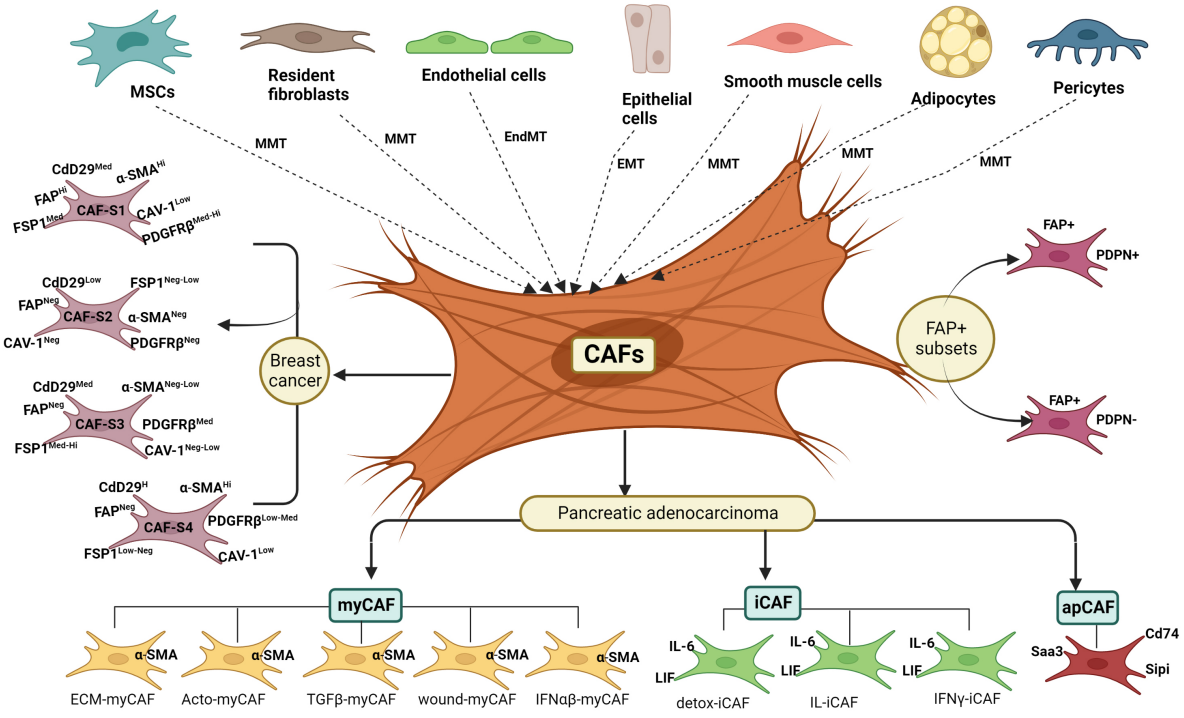

Figure 1. Origin and heterogeneity of cancer-associated fibroblasts. CAFs in the tumor microenvironment can be originated from MSCs, fibroblasts, adipocytes, pericytes, smooth muscle, endothelial and epithelial cells through the different trans-differentiation programs. Varieties of CAF subsets have been identified in cancer types of different tissue origins. The different subsets of CAFs show different functions and molecular features. CAF-S1 to CAF-S4 subsets are present in breast cancer. myCAF, iCAF and apCAF subsets are observed in PDAC. Several cancers exhibit overlapping populations of CAF subsets.

acquire mesenchymal phenotype and higher migration and contraction potentials[15]. This EMT program induced by platelet-derived growth factor (PDGF), TGFβ, EGF, etc., and is facilitated by the activation of mesenchymal specific transcription factors like Snail, Slug, Twist and FOXC2[15,42]. Tumor-associated endothelial cells might contribute to the CAF population [Figure 1]. A previous study has shown that endothelial cells are transdifferentiated into CAFs via endothelial to mesenchymal transition (EndMT) by losing expression of CD31 and gaining the expression of FSP-1 and α-SMA under the TGF-β stimulus[43]. In another study, auto/paracrine FGF2 has been shown to regulate the TGF-β-induced EndMT in tumor endothelial cells (TECs) via Elk1[44]. In a similar way, pericytes undergo pericytes to myofibroblast transition (PMT), a mesenchymal-to-mesenchymal transdifferentiation (MMT) process to generate CAFs in a microenvironment [Figure 1]. Hosaka *et al.* have recently reported that vascular pericytes are converted to CAFs by PDGF-BB to promote tumor growth and metastasis. PDGF-BB binds to PDGFRβ to induce the PMT program in pericytes[45]. CAFs are known to be generated from bone marrow-derived mesenchymal stem cells (MSCs) [Figure 1][46,47]. Recruitment of MSCs takes place in many pathological conditions such as tissue repair, inflammation, and neoplasia. MSCs are recruited from the bone marrow into the tumors and subjected to activation similar to many inflammatory cells by a plethora of cytokines and growth factors derived from tumor cells or activated stroma[46-48]. The cytokines involved in the activation are vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), PDGF, EGF, CCL2, etc[49-51]. The previous report showed that labeled MSCs have been shown to localize tumor mass and thus differentiate into pericytes and CAFs by acquiring de novo expression of characteristic markers such as α-SMA, FAP, tenascin-c and thrombospondin1[52]. Reports have shown that tumor-derived OPN induces MSC transformation into CAFs via MZF1-mediated TGF-β expression to promote more

aggressive local tumor growth and metastasis in breast cancer[53]. Even though different cell types contribute to CAFs, the major source of CAFs is resident fibroblasts. Resident fibroblasts in tumors undergo fibroblast to myofibroblast transition (FMT), a process of MMT to generate CAFs [Figure 1][18]. Various growth factors and cytokines, mechanical forces and cell-cell contacts regulate the FMT process[8,19,30]. Evaluation of the FMT process in the tumor microenvironment was initially achieved by Kojima *et al.* in the fibroblast and cancer cell co-implantation xenograft model. Their studies have revealed that autocrine activation of TGF-β and SDF-1 (CXCL12) signaling leads to the acquisition of myofibroblast phenotype in fibroblasts[54]. CBL (CBF1, Suppressor of Hairless, Lag-1) and p53 are considered tumor suppressors in different cancers. Silencing of CBL and p53 in fibroblasts leads to the attainment of CAF phenotype in normal fibroblasts[55]. Shimoda *et al.* have reported that TIMPless fibroblasts reflects the traits of CAFs. According to their studies, deletion of TIMP instigates the expression of α-SMA in fibroblasts and increases the migration and contraction potentials[56]. A recent study has described the role of nodal in the conversion of normal fibroblasts to CAFs with snail and TGF-β signaling pathway activation[57]. In addition, paracrine signaling cues derived from tumor cells play a major role in the acquisition of the CAF phenotype. Tumor-derived TGF-β is known to play a pivotal role in the generation of CAFs from the activation of resident fibroblasts[58]. Recruitment of fibroblasts into the tumor microenvironment is a prerequisite for CAF generation. A previous study has identified that tumor cell-derived Wnt7a recruits and activates fibroblasts to CAFs to promote tumor aggressiveness. Wnt7a exhibits a fibroblast-activating role by potentiating TGF-β receptor signaling and not relying on canonical Wnt signaling[59]. Epigenetic switch involving p300-mediated STAT3 acetylation induces the fibroblast activation to CAFs to support tumor invasion[60]. Mechanical forces and matrix stiffness induce several signaling pathways in the tumor microenvironment that are imperative for tumor aggressiveness. Matrix stiffness elevates the activity of Yes-associated protein (YAP) in nearby fibroblasts, thereby inducing the CAF phenotype in these cells[19]. Activated fibroblasts are reported to secrete various growth factors, cytokines such as SDF-1, IL-6, CXCL14, CCL5 and CCL7 and proteases such as MMP-2, MMP-9 and uPA to promote EMT in cancer[18].

HETEROGENEITY OF CAFS

The multipronged actions of CAFs on tumor cells probably reflect their heterogeneous population with context-dependent functions. Although CAFs are known to originate from resident fibroblasts, MSCs, endothelial cells, pericytes, epithelial cells, and adipocytes through trans-differentiation programs, CAF subsets have been represented as distinct cellular states rather than indicating their different cell origins. Costa *et al.* have identified four subsets of CAFs (CAF-S1, CAF-S2, CAF-S3 and CAF-S4) in breast cancer by combining the analysis of six CAF markers [Figure 1]. Higher levels of both CAF-S1 (FAP^{High} $CD29^{Med}$ $SMA^{Med-High}$ $FSP1^{Med}$ $PDGFR\beta^{Med-High}$ $CAV1^{Low}$) and CAF-S4 ($FAP^{Neg-Low}$ $CD29^{High}$ SMA^{High} $FSP1^{Low-Med}$ $PDGFR\beta^{Low-Med}$ $CAV1^{Low}$) subsets are reported in aggressive Her2+ and triple-negative breast cancer (TNBC)[61]. Moreover, accumulation of FAP^{High} CAF-S1 subset in early luminal breast cancers is associated with distant relapse[62]. In contrast, the CAF-S2 subset ($CD29^{Low}$ FAP^{Neg} $FSP1^{Neg-Low}$ $\alpha\text{-}SMA^{Neg}$ $PDGFR\beta^{Neg}$ $CAV1^{Neg}$) is highly accumulated in the luminal breast cancer subtype whereas CAF-S3 fibroblasts ($CD29^{Med}$ FAP^{Neg} $FSP1^{Med-High}$ $\alpha\text{-}SMA^{Neg}$ $PDGFR\beta^{Med}$ $CAV1^{Low}$) is observed in healthy tissues[61-63]. In addition, CAF-A (ECM remodeling) and CAF-B (myofibroblastic genes) are observed in colorectal cancer (CRC)[64].

Givel *et al.* have demonstrated fibroblast heterogeneity in high-grade serous ovarian cancers (HGSOC) by defining four subsets of CAFs (CAF-S1 to S4) as described in breast cancer[65]. Mesenchymal HGSOC consists of high CAF-S1 fibroblasts, which modulate immunosuppressive functions by increasing infiltration, survival, and differentiation of $CD25^{+}FOXP3^{+}$ T lymphocytes. SDF-1 β (CXCL12β) is specifically accumulated in the immunosuppressive CAF-S1 subset. Thus, their data highlight a CXCL12β-regulated stromal heterogeneity and immunosuppression in mesenchymal HGSOC[65]. The existence of CAF-S1 and

CAF-S4 molecular signatures has been validated in lung cancer[66] and head and neck cancer by leveraging publicly available single-cell data[67]. The presence of these two major CAF-S1/CAF-S4 myofibroblastic subpopulations was validated in different cancer types[68]. These data suggest the existence of both CAF-S1 and CAF-S4 myofibroblastic cells in distinct cancer types and across species.

Two subsets of CAFs were recently reported in pancreatic adenocarcinoma. One subset displays a matrix-synthesizing myofibroblastic phenotype termed myCAF, whereas another exhibits an immunomodulatory phenotype, inflammatory CAFs named iCAF [Figure 1] The CAFs proximal to the cancer cells show a myCAF phenotype with higher expression of α-SMA. Distal CAFs from the cancer cells express high levels of proinflammatory cytokines such as IL-6, G-CSF, CXCL1, and LIF and are defined as iCAFs[69]. IL-1 signaling induces iCAF signature, while TGF-β signaling controls myCAF signature by antagonizing the iCAF phenotype. Another study has demonstrated the two different subpopulations of CAF, named myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs), by employing a 3D co-culture system of PDAC *in vitro*[70]. Further, Elyada *et al.* reported the third subtype of CAFs, named antigen-presenting CAFs (apCAFs), using single-cell RNA sequencing (scRNA-seq) in PDAC tissues, and these are characterized by expression of H2-Aa, H2-Ab1 (encoding α, β -chains of MHC II), CD74, secretory leukocyte peptidase inhibitor (SLPI) and serum amyloid A3 (Saa3) genes [Figure 1]. Also, apCAFs possess antioxidant response and are regulated by IFN-γ signaling *in vivo*[71]. Furthermore, other studies also confirmed the apCAF classification based on the results obtained using scRNA-seq in pancreatic cancer[72,73]. In addition, transcriptomics study in normal pancreatic cells of KPP mice revealed that cells that express mesothelial signature also show the expression of MHC II genes, implicating that apCAF could be of mesothelial origin[74]. Later, apCAFs subtype has also been reported in breast and lung cancer[75-78]. Interestingly, apCAFs activate the CD4 + T lymphocytes, which implies that CAFs have antigen-presenting properties similar to other antigen-presenting cells such as macrophages, dendritic cells and B cells immunomodulatory functions. However,the study on orthotopic murine models of lung cancer showed that lung apCAFs are tumor-suppressive cells[77]. Another report has revealed the presence of two FAP+ subsets on the basis of PDPN expression [Figure 1][79]. The FAP^+ $PDPN^+$ fibroblasts show elevated expression of TGF-β signaling proteins and fibrosis-associated genes, whereas FAP^+ $PDPN^-$ cells displayed less expression of the same genes[68,71,79]. Moreover, a recent study further classified FAP^{High} CAFs into eight different clusters. Out of these clusters, five clusters (ECM-myCAF, Acto-myCAF, TGFβ-myCAF, wound-myCAF and IFNαβ-myCAF) belong to the myCAF subgroup and three clusters (detox-iCAF, IL-iCAF, IFNγ-iCAF) fall into the iCAF subgroup[68]. Therefore, CAFs possess multifaceted functions including tumor promotion and prevention based on the gene expression signatures.

CAFS REGULATE DRUG RESISTANCE BY MODULATING CANCER CELL SURVIVAL

Interestingly, cancer cells produce a variety of factors that recruit, activate, and help with the survival of CAFs; nonetheless, CAFs, in return, support cancer cell survival and proliferation by providing appropriate signaling factors which subsequently promote cancer cell resistance. Using mouse models of inflammation-induced gastric cancer, a study reported that at least 20% of CAFs are derived from MSCs of bone marrow, and show the expression of α-SMA, wingless-related integration site 5α (Wnt5α), IL-6, bone morphogenetic protein 4 (BMP4), and DNA hypomethylation. MSC-derived CAFs are recruited to dysplastic stomach in TGF-β and SDF-1α-dependent manner to promote tumor survival[80]. CAFs provide ovarian cancer cells resistance to cisplatin by secreting cisplatin-induced chemokine (C-C motif) ligand 5 (CCL5), which augments the phosphorylation of STAT3 and Akt in cancer cells. Thus, CAFs play a crucial role in promoting ovarian cancer cell growth by regulating STAT3/PI3K/Akt pathway [Figure 2A][81]. Interestingly, a heparin-binding growth factor, midkine (MK), derived from CAFs, provides cisplatin resistance to oral squamous cell carcinoma (OSCC), lung cancer, and ovarian cancer cells by enhancing the expression levels

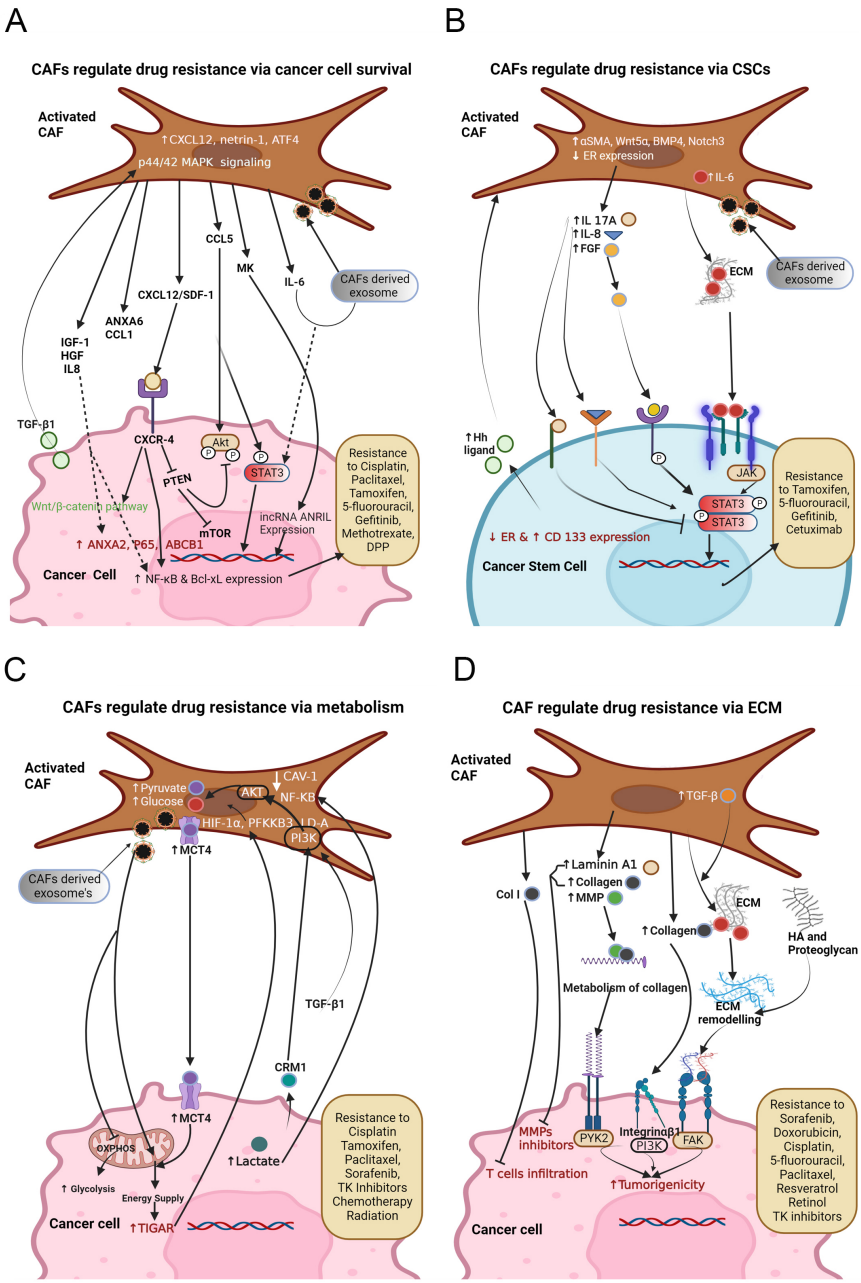

Figure 2. Fibroblast-mediated signaling in shaping the drug resistance in (A) CAFs promote cancer cells survival by secreting ANXA6, CCL1, CXCL 12, CCL5, MK, etc., and the exosomes containing lncRNA ANRIL, miR-196a, miR-103a-3p, miR-24-3p, microRNA-21, microRNA-148b-3p, LPP, CCAL, etc. Also, CAFs show high levels of netrin1, IL-6, and ATF4, which offer cancer cells gemcitabine resistance. TGF-β1 secreted by cancer cells acts on CAFs to attain 5-fluorouracil (5-FU) and tamoxifen (TAM) resistance. Inhibition of PTEN by CXCL12-CXCR4 binding promotes mTOR signaling and cancer proliferation. MK provides cisplatin resistance by ameliorating the expression of lncRNA-ANRIL; (B) CAFs induce stemness in cancer cells through the activation of STAT3 by IL8, IL6, and FGF and secreting exosomes containing miR-221 and H19 that leads to drug resistance, and STAT3 activation can be inhibited by IL-17A. High levels of α-SMA, Wnt5α, BMP4 and Notch3 in CAFs and low expression of ER in both CAFs and cancer cells are associated with enriching CSCs and drug resistance; (C) CAFs provide different nutrients to cancer cells. CAF-secreted factors rewire the cancer cell metabolism by the activation of autophagy, mTOR, and TIGAR and suppression of oxidative phosphorylation that leads to drug resistance; (D) CAFs promote drug resistance by ECM deposition. Activation of CAFs by TGF-β1 or other factors leads to excessive synthesis of ECM proteins such as laminin-A, collagen, and fibronectin. It also induces various MMPs, which leads to ECM modeling that blocks drug effects.

of the lncRNA-ANRIL [Figure 2A]. Therefore, targeting either MK production from CAFs or inhibiting the lncRNA-ANRIL in cancer cells could be the key to cancer treatment[82]. The mir-1-mediated expression of SDF-1 in CAFs induces the proliferation of lung cancer cells and chemoresistance via CXCR4-dependent pathway involving NF-κB and Bcl-xL[83]. CAFs are not only involved in promoting cancer cell viability but also induce EMT in response to drug treatments. An earlier study showed that CAFs promote EMT through the secretion of IGF-1 and HGF. These growth factors enhance the expression and phosphorylation of annexin A2 (ANXA2), which endorse the resistance to the EGFR-TKI (gefitinib) in NSCLC (HCC827 and PC9) cells-harboring EGFR activating mutations [Figure 2A]. Therefore, restricting the CAFs-induced EMT is necessary to subdue TKI-resistance[84]. EMT transcription factors such as Twist1 and Snail regulate the activation of CAFs in cancers[30,85]. Expression of Twist1 and Snail in CAFs also associated with the expression of several cytokines including SDF-1, CXCL1 and CCL2 which can regulate cell proliferation and survival[30,86]. Blanco-Gomez *et al.* demonstrated that loss of SNAI2 in CAFs limit the production of some cytokines such as SDF-1 and CXCL1, CXCL2, IFN-γ and IL-16, thereby impeding breast cancer cell proliferation and metastasis[86]. Thus, SNAI2 could be considered a therapeutic target to block both proliferation and EMT in tumor cells and cytokine production in CAFs.

Furthermore, CAFs elicit TGF-β-mediated EMT in ovarian cancer cells via IL-6-regulated JAK2/STAT3 pathway to inhibit cancer cell apoptosis and provide paclitaxel resistance[87]. CAF-secreted SDF-1 stimulates pancreatic cancer progression and aids in gemcitabine resistance by augmenting the expression of SATB-1[88]. A recent study showed that snail-positive fibroblasts facilitate chemoresistance to 5-fluorouracil and paclitaxel in colorectal cancer (CRC) by secreting CCL1 through the TGF-β/NF-κB signaling pathway[89]. In addition, CAFs upregulate the expression of the lipoma-preferred partner (LPP) in microvascular endothelial cells (MECs). The upregulated LPP upshots stress fiber formation and focal adhesion to further enhance the mobility and permeability of endothelial cells, which ultimately resulted in the enhancement of chemoresistance in ovarian cancer[90]. CAFs elevate human gastric cancer chemoresistance by higher expression of IL-8, which further regulates cell survival pathways including PI3K, Akt, IKb, p65, and ABCB1. Hence, IL-8 derived from CAFs involved in promoting chemoresistance in gastric cancer through NF-κB activation and upregulation of ABCB1 [Figure 2A][91]. Annexin A6 present in CAFs-derived extracellular vesicles plays an important role in inducing drug resistance and tubular network formation in gastric cancer by activation of FAK/YAP axis through the stabilization of β1 integrin on the surface of cancer cells[92]. Higher expression of activating transcription factor 4 (ATF4) in PDAC-derived CAFs promotes malignancy and gemcitabine resistance through TGF-β1/SMAD2/3 axis[93]. Intriguingly, TGF-β1 secreted from breast cancer cells activates CAFs in a paracrine manner, contributing to chemoresistance via activating p44/42 MAPK signaling pathway[94].

Higher expression of CXCL12 in interstitial CAFs contributes to EMT and cisplatin resistance in epithelial ovarian cancer (EOC) via CXCR4/Wnt/β-catenin pathway[95]. Additionally, this CAF-derived CXCL12 mediates inhibition of PTEN which is crucial for cancer cell proliferation [Figure 2A][96]. Likewise, CAFs are involved in offering cisplatin resistance in HNC cells by exosome-mediated transfer of miR-196a. Upon depletion of CAF-exosomal miR-196a, restoration of cisplatin sensitivity has occurred in HNC cells. Therefore, targeting miR-196a can serve as a better therapeutic approach to overcome cisplatin resistance in HNC cells[97]. Moreover, CAF-derived, highly expressed, exosomal miR-103a-3p accelerates cisplatin resistance and inhibits apoptosis in NSCLC cells by targeting BCL2- antagonist/killer 1 (Bak1)[98]. CAF-derived miR-24-3p containing exosomes promote cancer cell resistance to methotrexate by downregulation of the CDX2/HEPH axis in colon cancer[99]. The CAF-mediated transfer of exosome-containing lncRNA CCAL (colorectal cancer-associated lncRNA) to CRC cells initiates signaling towards gaining resistance to oxaliplatin via the β-catenin pathway. Interaction of CCAL with HuR (human antigen R, an RNA stabilizing

protein) leads to an increase in β-catenin, thereby providing oxaliplatin resistance in CRCs[100]. CAFs secreted IL-6/exosomal microRNA-21 (miR-21) induces the activation of STAT3 signaling to generate monocytic myeloid-derived suppressor cells (M-MDSCs) to further accelerate cisplatin (DDP). Therefore, inhibition of STAT3 signaling can restore cancer cells' drug sensitivity[101]. Transfer of CAF derived exosomes-containing miR-148b-3p to bladder cancer cells enhances tumor proliferation, EMT, metastasis, and drug resistance. Mechanistically, miR-148b-3p induces Wnt/β-catenin pathway by targeting PTEN[102]. Therefore, overexpression of PTEN might lead to suppression of metastasis, EMT, and drug resistance. CAFs respond to tamoxifen treatment by upregulating the expression of high mobility group box 1 (HMGB1) through GPR30/PI3K/AKT signaling. HMGB1 is involved in the induction of autophagy to increase resistance to tamoxifen in MCF-7 cells via an ERK-mediated manner[103]. Overall, the above reports suggest that CAF-secreted growth factors, chemokines and exosomes regulate drug resistance by inducing cell survival in different types of cancer.

CAFS REGULATE DRUG RESISTANCE BY MODULATING CANCER STEM CELLS

Cancer stem cells (CSCs) play a pivotal role in tumorigenesis, progression, and drug resistance. CSCs exhibit self-renewal and tumorigenic properties, which enable them to metastasize to distant sites, offering them a favorable environment. Moreover, the microenvironment around CSCs contributes a lot to fostering tumor growth through the modulation of CSC phenotype. The generation of CSCs through EMT is highly conditional on its surrounding matrix. This underscores the vital role of microenvironmental elements like CAFs and their secreted factors in shaping the renewal and maintenance of CSCs[104].

Stem cell pathways like Wnt signaling are important for maintaining stemness in non-cancerous cells of the colon. An interesting study suggested that cells surrounding the CSCs, especially myofibroblasts, maintain a higher Wnt activity in CSCs and manage to stimulate Wnt signaling in nearby differentiated tumor cells, thereby mending the stemness and tumorigenicity[105]. In response to the chemotherapy, CAFs express IL-17A, which helps with the self-renewal of cancer-initiating cells (CICs) to facilitate resistance to chemotherapies[106]. Therefore, targeting IL-17A signaling could impede CICs growth. Additionally, exosomes secreted by fibroblasts in response to chemotherapy are also known to promote the spheregenerating capacity and chemotherapy resistance in CSCs. To validate the role of CAF-derived exosomes in priming CSCs, blockade of exosome release by culturing CAFs in the presence of a specific inhibitor of neutral sphingomyelinase 2, GW4869 resulted in the restoration of chemosensitivity in CSCs[107]. Hence, blocking CAFs secretion can be an effective approach to increasing the efficacy of chemotherapy in combating cancers. Specifically, fibroblast-derived exosomes-containing Wnts promote Wnt activity in CRC cells to enhance chemoresistance[108]. The microvesicles (MV) derived from CAF, transfer miR-221 to CSCs to induce hormonal therapy (HT)-resistance. The overall loop of events, including CAFs release of MV, is associated with a reduction of ER expression followed by an increase in Notch expression in CSCs. The increase in Notch further elicits the reduction of ER levels and an increase in CD133 levels in CSCs [Figure 2B][109]. Moreover, CAFs are involved in supporting CSCs via combined activation of Wnt/β-catenin and HGF/Met signaling. CSCs regulate CAFs via secretion of Hh ligand, SHH to activate Hh signaling in a paracrine manner. In turn, CAFs secrete factors that help with CSC's self-renewal and expansion. The treatment of tumors with a Hh inhibitor, vismodegib, led to the reduction of CAF activation and CSC's expansion, thereby delaying the tumor formation and progression. Hence, targeting CAFs using Hh inhibitors can be an effective strategy for breast cancer treatment[110]. A study reported that Hh-stimulated CAFs contribute to the formation of chemo-resistant CSCs niche through the FGF pathway. Extracellular matrix rich in Hh-activated CAFs, FGF, and fibrillar collagen shape a conducive environment to foster a stem-like phenotype in triple-negative breast cancer (TNBC) cells[111]. Another study reported that HIF-1α and CAF-derived TGF-β2 crosstalk activate the expression of GLI2, a Hh

transcription factor, in CSCs, which further enhances the chemoresistance and stemness of CSCs[112]. Specifically, CD10 and GPR77-positive subsets of CAFs are associated with chemoresistance by creating a niche for enrichment of CSCs in the multiple cohorts of breast and lung cancer patients. The binding of C5a to GPR77 in CD10 + GPR77 + CAFs in an autocrine manner phosphorylates p65 which ultimately leads to the expression of IL-6, IL-8, CD10, and GPR77. Though GPR77 continues to be part of autocrine cycle, IL-6 and IL-8 regulate CSC's self-renewal[113]. Additionally, CAFs facilitate chemoresistance and stemness through the transfer of exosomal H19 to CRC. H19 produced by CAFs in CRC stroma mediates CSC phenotype by activating the β-catenin pathway[114]. The usage of MSC-derived fibroblasts (MSC-DF) has been reported for reciprocally studying the loss-of-function and gain-of-function of the Notch in the regulation of CSCs. It was found that MSC-DF Notch1-/- promoted the formation of spheroids in co-cultured melanoma cells, while MSC-DFN1IC+/+ (N1IC: Notch1 intracellular domain, an active form of Notch1) suppressed melanoma cell sphere formation capacity, thereby diminished tumor initiation properties. MSC-DFNotch1-/- could contribute to promoting stemness of melanoma cells by upregulating Sox2/Oct4/Nanog expression[115]. An earlier report revealed that IL-17A acts as a CSC-maintenance factor and helps with CSC renewal and invasion[116]. Intriguingly, epigenetic changes in CAFs also play a critical role in maintaining CSC-promoting capacity. A recent study revealed that the loss of H3K27me3 in CAFs leads to the expression of WNT5A, NOG, GREM1, and IGF2, which play an important role in maintaining stem cell niche, stromal-epithelial interaction, and cell growth[117]. Therefore, it is important to identify players involved in CAF-specific epigenetic changes in order to shed light on epigenetic-centered CAF-targeted therapies. Netrin-1 is highly expressed in CSC and known to regulate stemness. The higher expression of netrin-1 was also found in CAFs and associated with the increasing stemness in cancer cells, thereby mediating drug resistance. Inhibition of intercellular signaling between cancer cells and CAFs using Netrin-1-mAb suppresses the expression of CAF-borne cytokines such as IL-6 [118]. Taken together, CAF-regulated CSCs execute a crucial role in tumor initiation, maintenance, progression, and chemoresistance. Hence, finding a signaling messenger between CSCs and CAFs is indispensable for developing interventions to combat cancer

CAFS REGULATE DRUG RESISTANCE BY MODULATING CANCER CELL METABOLISM

The major energy sources for the survival of unconditionally growing tumor cells are glutamine (Gln) and glucose. Rewiring of cancer cell metabolism enables the survival of cancer cells by providing the building blocks/intermediates for the synthesis of nucleic acids, lipids and proteins. Tumor microenvironment (TME) or CAFs-mediated metabolic reprogramming of cancer cells regulates several signaling cascades that also result in drug resistance[119].

Notably, a study has shown that exosomes derived from CAFs can reprogram the metabolic machinery by their uptake into cancer cells[120,121]. These exosomes consist of intact lipids, amino acids, and intermediates of the TCA cycle[121]. Moreover, these exosomes inhibit mitochondrial OXPHOS, leading to increased glycolysis and Gln-dependent reductive carboxylation in cancer cells [Figure 2C]. It has been reported that CAFs predominantly express glucose uptake proteins in non-small cell lung cancer. Among these, glutamine-fructose-6-phosphate transaminase 2 (GFPT2) plays an important role in glycolysis, thus showing its significance in prognosis[122]. A previous study showed that epigenetic changes in CAF instigated a cascade of stromal-epithelial interactions to promote prostate cancer growth and resistance to androgen deprivation therapy (ADT). This study revealed that epigenetic silencing of a Ras inhibitor, RASAL3, in prostatic CAFs leads to oncogenic Ras activity that drives macropinocytosis-mediated glutamine synthesis. Interestingly, ADT further strengthens RASAL3 epigenetic silencing and glutamine secretion by CAFs. Therefore, high levels of glutamine have been found in prostate cancer patients after ADT[123].

Cancer cells, under glucose-deprived states, use aerobic glycolysis as their major energy source, known as the Warburg effect. Pyruvate kinase M2 (PKM2) is overexpressed in NSCLC cell lines and plays a role in mediating the Warburg effect which promotes resistance to cisplatin[124]. In another phenomenon, aerobic glycolysis in the cancer-associated stroma metabolically supports surrounding cancer cells, which is known as the reverse Warburg effect. This stromal-cancer metabolic coupling enables catabolite transfer to cancer cells for the generation of ATP, induction of proliferation, and reduction of cell death[125]. Interestingly, cancer cells educate stromal cells to display aerobic glycolysis that mediates multidrug resistance[126]. Moreover, in the majority of solid tumors, CAFs utilize more glucose and in turn release more lactate in comparison to normal fibroblasts[127]. Notably, cancer cells induce the Warburg effect in CAFs through activation of the PI3K/AKT pathway via translocation of nuclear G-protein-coupled estrogen receptor (GPER) in a chromosomal region maintenance 1 (CRM1)-dependent manner and abnormal activation of the GPER/cAMP/PKA/CREB signaling pathway[126]. Consequently, CAFs delivered lactate transporters to cancer cells, which increases drug resistance [Figure 2C]. In contrast, a study by Apicella *et al.* showed that cancer cells expressing EGFR- or MET exhibit increased glycolytic activity leading to elevated levels of lactate. The elevated levels of lactate educate CAFs to secrete higher levels of HGF via an NF-κB-dependent mechanism that subsequently leads to cancer cell resistance against TKI therapy[128]. In solid tumors, the hypoxic environment mediates chemoresistance, as its low pH affects the cytotoxicity of mitoxantrone, paclitaxel and topotecan[129]. It has been reported that under a hypoxic environment, CAFs secrete several factors that activate angiogenic (VEGF) and immunogenic (T-cell mediated cytotoxicity) signaling that is essential for tumor progression[130,131]. A study showed that hypoxia induces migration, type I collagen expression, and VEGF production in pancreatic stellate cells and mediates resistance to anticancer drugs[132]. Interestingly, it has been demonstrated that TGF-β expressed by other stromal cells activates fibroblasts and induces ECM production, and stimulates aerobic glycolysis and catabolic metabolism[133]. It has been observed in a cancer cell-fibroblast co-culture system, oxidative stress-induced autophagy leads to downregulation of caveolin-1 (CAV1) in CAFs and overexpression of TIGAR (TP53- Induced Glycolysis and Apoptosis Regulator) in adjacent cancer cells[134]. Downregulation of CAV1 in CAFs leads to mitochondrial dysfunction and glycolysis via HIF-1α and NF-kB signaling. Therefore, autophagic CAFs prevent cancer cell death by providing substrates for metabolic activity of cancer cells and upregulating TIGAR which confers resistance to tamoxifen-induced apoptosis and autophagy[135]. In addition, overexpression of TIGAR in cancer cells induces a glycolytic phenotype in CAFs and promotes the expression of HIF-1 α, PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3) and lactate dehydrogenase-A along with increasing glucose uptake [Figure 2C][134]. Overexpression of PFKFB3 has been shown to generate resistance to the BCR-ABL TKI in chronic myeloid leukemia (CML)[136]. Likewise, overexpression of HIF-1α along with glycolytic isoenzymes has been reported to be strongly associated with chemoresistance in different tumors[137,138]. Furthermore, CAFs-associated metabolic reprogramming also regulates epigenetic changes for the maintenance of the CAF active state; thereby, catabolic CAFs and anabolic cancer cells are metabolically coupled, contributing to the development of chemoresistant tumors[139-141]. In addition, the decrease in glucose concentration limits the synthesis of building blocks required for cell proliferation, leading to inhibition of cell proliferation. However, glucose starvation induces AMP-activated protein kinase (AMPK), which is an upstream factor of Hippo signaling; therefore, coupling of the metabolic pathway and Hippo signaling promotes drug resistance[142]. Metabolic coupling of CAFs and cancer cells is critical for regulating resistance to different therapeutic regimens. Therefore, devising therapeutic interventions to disrupt the metabolic coupling of CAFs and cancer may open new avenues for cancer treatment. However, how cancer cells educate CAFs to trigger resistance-mediating pathways is still poorly known.

CAFS REGULATE DRUG RESISTANCE BY MODULATING ECM

CAFs orchestrate tumor promotion and drug resistance by increasing matrix stiffness via augmenting the expression of ECM components such as hyaluronic acid (HA) and collagens[143]. Both HA and collagen are known to withstand tensile stress and the activity of collagen receptor, integrin α11β1, is associated with matrix stiffness. It has been reported that in NSCLC, CAFs can promote the stiffness of interstitial collagen by enhancing the expression of integrin α11, leading to tumor progression[144]. A recent study demonstrated that collagen secreted by CAFs acts in a paracrine manner to regulate the resistance to microtubule-directed chemotherapeutic drugs through integrinβ1/PI3K/AKT pathway in breast cancer [Figure 2D][145]. The dense number of CAFs was observed to play an important role in desmoplastic reactions in PDAC[146]. In addition, the CAFs-derived intense desmoplastic response in fibrotic tumors builds a dense ECM barrier that reduces the delivery of any drug to the tumor cells. In breast cancer patients, a progressively noncompliant fibrotic stroma limits the chemotherapeutic efficiency of doxorubicin[147]. Also, based on desmoplastic scores, CAFs have been divided into high desmoplastic CAFs (HD-CAFs) and low desmoplastic CAFs (LD-CAFs). The NSCLC patients with HD-CAFs showed a high collagen matrix remodeling rate which played a critical role in tumor progression via regulation of invasion and growth[148]. Likewise, HD-CAFs (alpha-smooth muscle actin positive myofibroblasts) in PDAC are significantly involved in the secretion of type I collagen (Col1) which plays a role in restricting drug delivery and impeding T cell infiltration[149].

Further, CAFs produce metalloproteinases (MMPs) that enhance tumor invasion by matrix remodeling[150]. CAFs employ MMP endopeptidases for the degradation of basement membrane (BM) proteins[151]. A previous study demonstrated the role of CAFs in BM stretching that facilitates the migration of CAFs and tumor cells into the bloodstream and metastasizes to distant organs. Intriguingly, the alternative CAF-dependent mechanism where BM shows a high tendency of stretching due to low expression of type IV collagen and laminin and rendering the head and neck tumor cells resistant to MMP inhibitors[152]. Another study reported that MMPs derived from CAFs are involved in tamoxifen resistance through EGFR and PI3K/AKT pathways in breast cancer [Figure 2D][153].

In addition to this, CAFs also secrete other factors such as caveolin-1 and podoplanin (PDPN), which are associated with wound responses[154]. The expression of a lymphatic vessel marker, PDPN, by stromal CAFs has been reported as a prognostic indicator in different cancer types. For instance, Yoshida *et al.* have demonstrated that lung adenocarcinoma cells, when co-cultured with the PDPN+ CAFs, show greater drug resistance in comparison to normal cells. Similarly, in postoperative recurrence, PDPN+ patients possess a lower treatment response to EGFR-TKIs compared to PDPN- patients, suggesting the implication of PDPN+ CAFs in regulating drug resistance[155]. The above information indicates that dense ECM produced by CAFs acts as a mechanical barrier for drug delivery and immune cell infiltration, and it also provides the source for matrix remodeling enzymes and signaling molecules that further impede the efficacy of anticancer therapeutics. Therefore, ECM-depletion strategies might pave the way for the development of next-generation anticancer drugs.

TARGETING CAFS WITH NATURAL PRODUCTS AND ANALOGS

The utilization of natural products for the treatment of different diseases is indeed cost-effective and minimally invasive[156-158]. Numerous anticancer products have been isolated and characterized from natural sources. Apart from directly showing anticancer activity, they also provide leads for developing potent therapeutic drugs[159].

CAFs have emerged as an intriguing therapeutic target in cancer due to their indispensable role. CAFs and cancer cells reciprocally crosstalk to regulate several aspects of cancer progression and several growth

factors and cytokines act as a messenger in this crosstalk [Table 1]. Hence, targeting CAFs using natural products will be advantageous for reducing the burden of cancer as well as overcoming deleterious effects caused by drug treatments in cancer patients. Polyphenols present in green tea have potential anticancer activities. Treatment with tea polyphenol, epigallocatechin-3-gallate (EGCG), decreases the serum levels of HGF and VEGF in prostate cancer patients. Since HGF and VEGF are mostly secreted by stromal myofibroblasts in the tumor microenvironment, EGCG can prevent myofibroblast differentiation in prostate cancer[160]. Gray *et al.* have demonstrated that combinational treatment of EGCG and another polyphenol, luteolin, synergistically reduces TGF-β-induced myofibroblast differentiation and fibronectin synthesis by impeding ERK and RhoA signaling in prostate fibroblasts[161]. CAFs are one of the key contributors in introducing drug resistance to various anticancer chemotherapeutic agents. It was shown that CAFs are involved in acquiring the resistance to cisplatin by expressing Wnt16[162]. Quercetin is a member of flavonoids that show antioxidant properties. In this regard, Hu *et al.* have found that quercetin significantly inhibits Wnt16 expression in activated fibroblasts, thereby improving the anticancer effects of cisplatin[163]. Various reports have shown that curcumin, a phyto-polyphenol pigment found in spice turmeric, exhibits antioxidant, anti-inflammatory, neuroprotective, and anticancer activities against various cancers[164]. In addition, curcumin also regulates the TME of CAFs. An earlier study has shown that curcumin induces DNA damage-independent and safe-senescence in CAFs by upregulating p16. It also decreases the expression of α-SMA and reduces the migration and invasion potentials of CAFs. Furthermore, curcumin abolishes tumorigenic potentials of CAFs by downregulating the expression of IL-6, SDF-1, MMP-2, MMP-9 and TGF-β[165]. In the pancreatic cancer model, curcumin suppresses the expression of α-SMA, vimentin, and secretory factors in CAFs, thereby inhibiting EMT and metastasis of cancer cells[166]. The above reports indicate that curcumin might have therapeutic potential for impeding the crosstalk between cancer cells and CAFs.

Fraxinellone (FRA) is a member of the limonoids family. Several studies have reported the medicinal properties of FRA, including neuroprotective, antifibrotic, anti-inflammatory, and antitumor functions[167]. An earlier study has reported that FRA regulates TGF-β signaling in fibrotic liver disease[168], which hints therapeutic potential of FRA in treating cancer, as both are characterized by the accumulation of myofibroblasts. A recent report demonstrated that FRA-loaded nanoparticle inhibits the CAF phenotype by impeding TGF-β signaling in PDAC[169]. Mangostin (MG) is a xanthone and exhibits several medicinal properties such as antibacterial, antifungal, antioxidant, anti-inflammatory, anticancer, and cardioprotective effects[170]. Studies have demonstrated that MG displays antitumor activities by inducing apoptosis and inhibiting angiogenesis, ECM modification, and EMT[171]. A study has recently demonstrated MG's effect in regulating the tumor stroma. A nano-formulated MG suppresses TGF-β/Smad signaling leading to CAF inactivation and ECM reduction in pancreatic cancer[172].

Cyclopamine is a steroid alkaloid and the first small-molecule inhibitor of the Hh signaling pathway[173]. Several reports show that Hh signaling displays a critical role in proliferation and tumor-promoting functions indicating the potential of cyclopamine to reprogram CAFs[174,175].

Co-delivery of cyclopamine and paclitaxel nanoparticles in pancreatic cancer modulates tumor stroma by disrupting cancer-stroma crosstalk and reducing ECM stiffness[174]. Chrysin is classified as a member of the flavonoids, which exerts multiple biological effects including antidiabetic, antioxidant, hepatoprotective, anti-inflammatory, and anticancer activities[176]. Chrysin induces apoptosis in colorectal and gastric cancer cells[177,178]. A synthetic analog of chrysin named 8-bromo-7-methoxy chrysin inhibits the activation of hepatic stellate cells to CAFs, thereby reducing the stemness of cancer cells by impeding IL-6 and HGF signaling[179]. We have listed several natural or synthetic drugs for targeting CAFs in Table 2.

Table 1. The interactions between CAFs and cancer cells

Source cells	Factors	Recipient cells	Biological effect of released factors	Affected signaling pathways	Reference
CAFs	CXCL12	Breast cancer	OPN-CAF-derived CXCL12 promotes EMT	ERK1/2 and AKT	[8]
CAFs	CCL5	Ovarian cancer	Cisplatin resistance	STAT3/PI3K/Akt pathway	[81]
CAFs	SDF-1	Lung cancer	Chemoresistance	mir-1/SDF-1/CXCR4/NF-κB/Bcl-xL	[83]
CAFs	IGF-1, HGF	Lung cancer	EMT in NSCLC	IGF1/HGF/ANXA2	[84]
CAFs	IL-6	Ovarian cancer	Paclitaxel resistance	TGFβ/JAK2/STAT3/IL6 pathway	[87]
CAFs	SDF-1	Pancreatic cancer	Gemcitabine resistance	SDF-1/CXCR4/SATB-1 pathway	[88]
CAFs	CCL1	Colorectal cancer	Chemoresistance to 5-FU and paclitaxel	TGF-β/NF-κB pathway	[89]
CAFs	IL-8	Gastric cancer	Cisplatin resistance	NF-κB pathway	[91]
Breast cancer	TGF-β1	CAFs	5-FU and tamoxifen (TAM) resistance	p44/42 MAPK pathway	[94]
CAFs	CXCL12	Ovarian cancer	EMT and cisplatin resistance	CXCR4/Wnt/β-catenin pathway	[95]
CAFs	miR-24-3p	Colon cancer	Resistance to methotrexate	CDX2/HEPH axis	[99]
CAFs	miR-148b-3p	Bladder cancer	EMT, metastasis, and drug resistance	Wnt/β-catenin pathway	[102]
CAFs	IL-17A	Cancer-initiatingcells	Resistance to chemotherapies	IL-17A signaling pathway	[106]
CSCs	SHH	CAFs	CSCs expansion	Wnt/β-catenin signaling pathway	[109]
CAFs	TGF-β2	CSCs	Chemoresistance and stemness of CSCs	HIF-1α/TGF-β2-GLI2 pathway	[112]
CAFs	H19	CRC	Elevates stemness in CRCs	miR-675-IGFR signaling circuit & β-catenin pathway	[114]

COMPOUNDS UNDER CLINICAL TRIAL

There are several compounds under clinical trials for targeting the CAFs or CAF-mediated effects for the management of cancer[198]. Notably, losartan, a small molecular inhibitor, sold under the brand name Cozaar, is used for the treatment of diabetic kidney disease, heart failure, and left ventricular enlargement. It inhibits the angiotensin receptor by blocking binding of angiotensin II. It suppresses collagen and hyaluronan levels, which are known to be synthesized by CAFs, and it is currently under clinical trial[199]. Defactinib is a small molecular inhibitor of FAK, available under brand names, VS-6063 and PF-04554878. It is under phase II clinical trial for the treatment of patients with KRAS-mutant NSCLC and is known to target downstream signaling of integrins and interfere with CAF actions[200]. Vitamin D receptor agonist, paricalcitol, is under phase I and II studies to examine the benefit of it in combination with gemcitabine and nab-paclitaxel for the treatment of pancreatic cancer as it is known to normalize pancreatic stellate cells[201]. Galunisertib is a pharmacological small molecule inhibitor of the TGF-β signaling. Treatment with Galunisertib interferes with TGF-β signaling induced activation of CAFs and immunosuppression. Studies of phase I, and phase II trials are underway to compare the overall survival (OS) of patients with pancreatic cancer after the treatment with a combination of Galunisertib and gemcitabine as compared to gemcitabine alone[202,203]. IPI-926 (saridegib) and vismodegib are small molecular inhibitors that target Hh signaling and reduce CAF activation, and are under clinical trials[204,205]. Several compounds for targeting CAFs are under clinical trials [Table 2].

CONCLUSIONS AND FUTURE DIRECTIONS

Stromal fibroblasts constitute a major component of tumor microenvironment. Fibroblasts can thrive in severe adverse conditions because of their intrinsic survival programs and cellular plasticity. Due to this ability, they can withstand insults from anticancer regimens. Simultaneously, these cells activate cell

Table 2. Drugs targeting CAFs for management of cancer

Drug natural	Type of cancer	Target/Interference with		Mechanism	Reference
		CAFs	CAFs functions		
EGCG	Colorectal		↓Glycolytic activity	↓PFK	[180]
Conophylline	HCC	↓α-SMA	↓IL6, IL8, CCL2, angiogenin, OPN	↓GPR68	[181]
α-mangostin	Pancreatic	↓α SMA/FAP/fibronectin	↓fibronectin/collagen	↓TGF-β pathway/Smad	[172]
Fraxinellone	Pancreatic	↓α SMA/FAP/fibronectin	-	↓TGF-β pathway	[169]
Triptonide	Gastric	-	↓IL-6, ↑TIMP2	↓MiR-301a ↑MiR-149	[182]
Chrysin	Liver	-	↓IL-6, HGF	-	[179]
Paeoniflorin	Gastric	-	↓IL-6	↑MicroRNA149	[183]
Resveratrol	CCA	-	↓IL-6	-	[184]
Minnelide	Pancreatic	↓α-SMA	↓Collagen/fibronectin/periostin/hyaluronan/ MMP2/MMP9	↓TGF-β RAR/RXR pathway	[185]
Cyclopamine	Pancreatic	-	↓LOX/hyaluronan	↓Hh pathway	[186]
Polyphyllin I	Gastric	↓FAP	↓HGF	-	[187]
Curcumin	Pancreatic	-	↑E-cadherin, ↓vimentin	-	[166]
Astragaloside IV	Gastric	-	↓M-CSF, ↑TIMP2	↑microRNA-214 ↓ microRNA-301a	[188]
Synthetic drugs					
Ursolic acid	PTC	-	↓CXCR4, CXCR7	-	[189]
CFH/OM-L	Hepatic	-	↑E-cadherin, ↓vimentin, N-cadherin, snail protein	-	[190]
Nintedanib	Hepatic	↓α-SMA	IL-6, IL-8	-	[191]
BTZ and PST		-	-	↑Caspase-3 mediated apoptosis	[192]
Drugs under clinical trial					
JNJ-42756493	NSCLC, Urothelial, Esophageal	↓FGFR ↓TK	-	-	[193]
Plerixafor	Pancreatic, Ovarian and Colorectal	-	↓CXCR4	-	[194]
PEGPH20 and MK-3475	PDAC	-	↓Hyalouronan	-	[195]
AT13148	Advanced solid tumors	-	↓ROCK	-	[196]
IPI-926 and Gemcitabine	Pancreatic	-	-	↓Hh pathway	[197]

α-SMA: A-smooth muscle actin; BTZ: bortezomib; CCA: cholangiocarcinoma; CCL2: C-C motif chemokine ligand 2; CFH/OM-L: CFH peptide (CFHKHKSPALSPVGGG)-decorated liposomal oxymatrine; EGCG: epigallocatechin-3-gallate; FAP: fibroblast activation protein alpha; HCC: hepatocellular carcinoma; Hh: hedgehog; MMP2: matrix metalloproteinase 2; OPN: osteopontin; PFK: phosphofructokinase; PTC: papillary thyroid carcinoma; PST: Panobinostat; TIMP2: tissue inhibitor of metalloproteinase 2.

resistance programs in cancer cells in response to inhibitory effects caused by the treatment modalities. Therefore, it is very important to understand the intrinsic survival programs and cellular plasticity that endure these cells from chemotherapy insults. Since the reciprocal interactions between the cancer cells and CAFs through soluble factors play a crucial role in orchestrating drug resistance programs, it is critical to profile CAF-derived secretome in response to chemotherapy to identify druggable targets. The CAF-mediated direct cell-cell and cell-matrix interactions also shape drug resistance in cancer cells. Hence, investigating the ECM-remodeling and changes in cell adhesion molecules triggered during the

development of drug resistance might provide insights into target proteins. Moreover, the double-edged sword effect of CAF signaling has been well recognized in tumor progression and drug resistance. Dissecting CAF-signaling and its function enables comprehensive identification of signaling pathways required to instigate drug resistance programs and facilitates targeting drug-resistant inducing cues and sparing the inhibitory cues.

DECLARATIONS

Authors' contributions

Conceptualized, wrote the significant part, and edited manuscript: Butti R
Wrote parts, made figures and edited manuscript: Khaladkar A, Bhardwaj P, Prakasam G

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All authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

Review

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Targeting MYC-driven lymphoma: lessons learned and future directions

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Abstract

MYC plays a central role in tumorigenesis by orchestrating cell proliferation, growth and survival, among other transformation mechanisms. In particular, MYC has often been associated with lymphomagenesis. In fact, MYC overexpressing lymphomas such as high-grade B-cell lymphoma (HGBL) and double expressor diffuse large B-cell lymphomas (DLBCL), are considered addicted to MYC. In such a context, MYC targeting therapies are of special interest, as MYC withdrawal is expected to result in tumor regression. However, whether high MYC levels are always predictive of increased sensitivity to these approaches is not clear yet. Even though no MYC inhibitor has received regulatory approval to date, substantial efforts have been made to investigate avenues to render MYC a druggable target. Here, we summarize the different classes of molecules currently under development, which mostly target MYC indirectly in aggressive B-cell lymphomas, paying special attention to subtypes with MYC/BCL2 or BCL6 translocations or overexpression.

Keywords: High-grade B-cell lymphoma, double expressor lymphoma, MYC, MYC therapies

INTRODUCTION

High-grade B-cell lymphomas

Lymphomas are very heterogeneous neoplasms that originate from the clonal expansion of B cells, T cells or natural killer (NK) cells[1]. They are divided into Hodgkin (HL), which represents 10% of the cases, and Non-Hodgkin (NHL), accounting for the remaining 90%[2]. In 2020, over half a million new cases of NHL were estimated globally[3]. NHLs are further subdivided according to their cell lineage, maturity of the cells and aggressiveness, following an evidence-based classification. Elaborated by the World Health Organization (WHO), such a classification has served as a global reference for the diagnosis of lymphoid neoplasms since its third edition in 2001 up to the current fifth edition published in 2022 (WHO-HAEM5)[4]. Additionally, the International Consensus Classification (ICC) recently published a report suggesting that the algorithm for the diagnosis of aggressive B-cell lymphomas, based on the current combination of morphology, immunophenotype, Epstein-Barr encoding region (EBER) *in situ* hybridization, fluorescent *in situ* hybridization (FISH) and B-cell clonality analysis, should be replaced by molecular genetic classification, based on mutational profile, somatic copy number alterations and structural variants, able to distinguish seven genetic subtypes with apparent clinical relevance[5].

The most common aggressive NHLs in Western countries are diffuse large B-cell lymphoma (DLBCL), Mantle Cell lymphoma (MCL) and Burkitt lymphoma, which account for 31, 6 and 2% of adult cases, respectively[6]. Owing to their unique genomic features, biological behavior and poor clinical prognosis, the subtypes of DLBCL, formerly known as double-hit (DHL) and triple-hit (THL) lymphomas, were classified in 2016 as a new category, termed "high-grade B-cell lymphoma" (HGBL), with translocations involving *MYC* and *B-Cell Lymphoma* 2 (*BCL2*) and/or *B-Cell Lymphoma* 6 (*BCL6*). In WHO-HAEM5, tumors with *MYC* and *BCL2* rearrangements are named DLBCL/HGBL *MYC*/*BCL2*, while HGBL-DH-*BCL6* represents a separate entity. HGBL-DH-*BCL6* are biologically less distinctive, hence considered genetic subtypes of either DLBCL, not otherwise specified (NOS) or HGBL, NOS. Complementarily, ICC considers double-hit (DH)-HGBL to comprise two entities: HGBL with *MYC* and *BCL2* rearrangements, with or without *BCL6* rearrangement (HGBL-DH-*BCL2*), and a provisional entity, HGBL-DH-*BCL6*, with *MYC* and *BCL6* rearrangements[7]. In terms of mutational and gene expression profiles, DLBCL/HGBL *MYC*/*BCL2* exhibit a mutational signature closer to that of follicular lymphoma (FL), including *Cyclic adenosine monophosphate Response Element Binding Protein* (*CREBBP*), *BCL2*, *Lysine Methyltransferase 2D* (*KMT2D*), *MYC, Enhancer of Zeste Homolog* 2 (*EZH2*) and *Forkhead box protein O1*(*FOXO1*), while showing a gene expression profile similar to centroblasts of the germinal center (GC) dark zone. In contrast, HGBL-DH-*BCL6* less frequently shows a Germinal center B cell-like (GCB) immunophenotype, is cytogenetically less complex and exhibits impairment of *E2F* targets, but not of the *TP53* and *MYC* signaling pathway, characteristic of DLBCL/HGBL *MYC*/*BCL2*[5,7].

DLBCL co-expressing MYC and BCL2, also known as double expressor lymphomas (DELs), are associated with shorter overall survival (OS) and Progression-Free Survival (PFS). Unlike DHL, whose cell-of-origin (COO) is primarily GCB, DELs are typically activated B cell-like (ABC)[8]. Further epidemiologic analysis of DHL/THL and DEL cases reveals an incidence of 2%-12% and 19%-34% among the DLBCL, respectively[9]. Given their dismal outcome, in the last few years since the WHO revised the classification, several reviews in the literature have pinpointed these diseases as an unmet medical need[8,10-13].

In terms of therapeutic opportunities, DLBCL follow standard rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone (R-CHOP) therapy. Although there is no widely accepted standard approach to manage HGBL with *MYC* and *BCL2*/*BCL6* rearrangements, retrospective comparisons show that DHL cases do better when treated with dose-intensive approaches compared to R-

CHOP, like dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, hydroxydaunorubicin, and rituximab (DA-EPOCH-R), or cyclophosphamide, doxorubicin, vincristine, high-dose methotrexate/ifosfamide, etoposide, and high-dose cytarabine (R-CODOX-M/R-IVAC). Others have proposed combining it with targeted agents against MYC or BCL2, like lenalidomide, an immunomodulatory agent, which in some contexts causes MYC downregulation, or venetoclax (also known as ABT-199), a highly selective inhibitor of BCL2, as promising new approaches[14].

Burkitt lymphoma

Burkitt lymphoma (BL) is the most common NHL in children and young adults (representing 40%-50% of pediatric NHL)[15]. Its definition has remained unchanged both in WHO-HAEM5 and ICC. It is a very aggressive tumor, characterized by *MYC* rearrangement with immunoglobulin genes and mutations in *Transcription Factor 3* (*TCF3*) or its negative regulator *Inhibitor of Protein Binding 3* (*ID3*), as well as coding mutations that affect the B-cell receptor (BCR), G Protein-coupled receptor (GPCR) and Phosphatidylinositol 3-Kinase (PI3K) signaling pathways[4]. It is associated with a GCB phenotype and is considered highly proliferative[7]. However, the ICC replaced the provisional entity Burkitt-like lymphoma with 11q aberration with Large B-cell lymphoma with 11q aberration, because it is more resemblant to DLBCL than BL[5].

In this context, treatment options, although different between adults and children, are mainly reduced to combinatorial chemotherapeutic regimens, like those used in other subtypes of lymphoma[16].

MYC, BCL2 and BCL6 deregulation and diagnosis of HGBL

The MYC family of oncoproteins is found to be deregulated in up to 70% of human cancers[17]. In physiological conditions, its expression is tightly regulated at all levels, from transcription to post-translational modifications[18]. Nonetheless, many of the genetic alterations present in cancer uncouple *MYC* expression from the usual regulatory constraints, either by constitutive activation of signal transduction pathways [e.g., Neurogenic locus notch homolog protein 1 (Notch), Wingless-related integration site (Wnt) and receptor tyrosine kinases (TKs)], or direct alterations of *MYC*, such as point mutations leading to protein stabilization, amplifications or translocations[18,19]. However, MYC overexpression is not always sufficient to drive tumorigenesis and often requires additional mutations, especially in cases where *MYC* expression induces not only proliferation, but also senescence or apoptosis[20,21]. Hence, these fail-safe mechanisms need to be disabled for MYC to exert its full pro-tumorigenic function.

Another family of proteins frequently mutated in cancer is BCL2, informally known as "guardians of cell death"[22]. These protein members display opposing functions to either induce or block the intrinsic apoptotic pathways. Physiologically, a delicate balance between pro- and anti-apoptotic proteins must be maintained[23]. However, resistance to apoptosis is one of the best-described cancer hallmarks. Thus, in the cancer context, it is common to observe an increase in the expression of anti-apoptotic family members, like BCL2, which happens to be one of the main contributors to B-cell lymphomagenesis[24]. As with MYC, BCL2 can be deregulated through various mechanisms that include (i) indirect ones, such as the activation of signaling pathways [e.g., Phosphatidylinositol 3-Kinase/Protein Kinase B (PI3K/AKT) or Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB)] and loss of Myeloid Leukemia 1 (MCL-1), and (ii) direct lesions, such as somatic mutations (restricted to FL with increased risk of transformation to DLBCL), amplifications, hypomethylation of the gene promoter or translocations with immunoglobulins[25]. Interestingly, BCL2 alone is also insufficient to induce full tumor development. Nevertheless, the cooperation of MYC and BCL2 does unleash the neoplastic transformation by simultaneously removing the brakes of cell growth and promoting cell survival[26,27].

BCL6 belongs to a family of transcription factors key for the germinal center reactions. It functions by recruiting corepressors that block the transcription of over 1,000 genes involved in the proliferation and survival of healthy germinal center B-cells, including cell cycle checkpoints and DNA damage repair-related genes[28,29]. Notably, both MYC and BCL2 are targets of BCL6, and in normal conditions, this transrepressor downregulates both proteins[28]. However, there are various direct and indirect mechanisms for BCL6 deregulation. For instance, BCL6 cross-talks with proteins involved in chromatin modifications, such as EZH2, CREBBP and KMT2D. Hence, mutations in these genes can add up to an imbalanced BCL6 activity[30]. More directly, reduced phosphorylation leading to protein stabilization, as well as translocations or mutations on the first non-coding exon, also result in altered expression of BCL6[30].

Importantly, 5%-15% of large B-cell lymphomas bear a translocation in MYC and BCL2 or BCL6, and 20%-30% of the cases are double expressors. Double expressors are defined in most studies as displaying ≥ 40% MYC positive and ≥ 50%-70% BCL2 positive cells. Such elevated expression of both proteins is caused by mechanisms other than gene rearrangements, such as amplifications, point mutations or oncogenic activation of signaling pathways[31,32].

Diagnosis of DHL/THL and DEL

MYC/BCL2 DHL is described as the most common type of DHL (at least 65% of the diagnosed cases), followed by MYC/BCL2/BCL6 THL, being MYC/BCL6 DHL the rarest[33,34]. The technique of choice to detect rearrangements in MYC(8q24), BCL2(18q21) and/or BCL6(3q27) is Fluorescent In situ Hybridization (FISH). Remarkably, even though most of the MYC/BCL2 cases show overexpression of both proteins, as many as 20% of these patients lack such correlation, with lower MYC levels typically associated with better outcomes. This is also true for the other DHLs and THLs, in which the genomic translocations generally result in high levels of the respective proteins and a worse prognosis[32].

Accurate diagnosis would appear to require the screening of all DLBCL patients by FISH[35]. While this has been agreed to be the best practice, as referred to in the WHO classification, the technique is not always routinely available. In Europe, only 40% of the countries have this capability, while another 40% rely only on referral hospitals[36].

It is important to distinguish DHL from DEL, which is far more common and is also related to poor prognosis. Of note, DELs are usually determined using immunohistochemistry (IHC), a less robust technique than FISH and, thus, more susceptible to variability. Besides, different cut-offs are defined between studies, although most accepted values are 40% positive cells for MYC, 50%-70% for BCL2 and 60% for BCL6[36,37]. Nowakowski and Czuczman raised the question of whether successful treatments for DHL would equally succeed for DEL, given the inherent differences between their COOs[37]. Interestingly, Ennishi *et al.* discovered a 104-gene double-hit signature (DHITsig) able to distinguish a subpopulation of patients likely to respond to R-CHOP and identify tumors with potential targetable vulnerabilities. DHITsig-positive tumors, as expected, show more frequent MYC and BCL2 alterations than DHITsig-negative tumors, even though only one-half of the cases harbor MYC/BCL2 rearrangements[38]. Additionally, Sha *et al.*[39] described a molecular high-grade (MHG) subgroup of DLBCL patients from a clinical trial investigating the addition of bortezomib to standard R-CHOP therapy. Such a subgroup encompassed most patients with DHL, extending the molecular identification to more than double the size of this poor-prognosis group. The authors suggest that MHG patients could benefit from intensified chemotherapy or novel targeted therapies[39]. Interestingly, in an independent study from the same authors, Cucco *et al.* identify a novel association between MHG-DLBCL with *MYC* hotspot mutations that lead to its stabilization and enhance its transforming capacity[40]. Studies like these emphasize the relevance of expanding our knowledge of the molecular mechanisms underlying the disease to best adequate the treatment.

Given the well-established role of MYC protein in driving lymphoma progression and the numerous preclinical studies in which suppression of MYC activity triggered regression of many tumor types considered MYC-driven[41-44] - including those where MYC is not deemed the initiating oncogenic lesion[45,46], it is clear that MYC inhibition could represent an effective treatment avenue.

In this review, we summarize the active preclinical and early-phase clinical research exploring novel approaches for the treatment of HGBL, focusing on MYC targeting therapies.

MYC-targeted therapies

In the last few years, several novel approaches have been explored to improve the poor outcome observed in lymphoma patients with the subtypes of DHL or THL, as well as DEL, even though the latter is not considered an independent entity. Many of the targeted therapies proposed for these specific tumors target MYC only indirectly, causing its downregulation in most cases. Here we list the different compounds classified by their primary mechanism of action [Table 1] and refer to the preclinical and clinical data available for each of them.

Bromodomain extra-terminal inhibitors

Bromodomain extra-terminal (BET) inhibitors have been a promising class of drugs in the cancer field for the past 10 years[47]. Inhibition of these transcriptional regulators can result in the silencing of MYC expression when it is under the control of super-enhancer elements[48]. A first-generation BETi, JQ1, for instance, was used in DHL/THL cells and showed the ability to slow down cell growth and induce apoptosis in a dose-dependent manner, and increased the therapeutic effect of the BCL2 inhibitor venetoclax[49,50]. A similar combination was later tested with another BETi, CPI203, which achieved simultaneous downregulation of MYC and Bcl-2-related protein A1 (BFL-1), overcoming the emergence of resistance to venetoclax both in DHL cultures and tumor xenografts[51].

Notably, it was expected that high MYC levels would be predictive of enhanced sensitivity to BETi. However, this is not always the case. For example, independent preclinical studies of different hematologic malignancies evidenced that MYC amplification failed to predict the sensitivity to the BETi OXT015[52]. Moreover, in a Phase I clinical trial with prostate cancer patients, there was a lack of correlation between the reduction in MYC levels and the response to the pan-BET inhibitor Zen-3694[52]. Similarly, Li *et al.*[53] demonstrated that several DHL/THL cell lines were as sensitive to various BETis (I-BET-762, JQ1 and OTX015) as U2932, a lymphoma cell line with no MYC rearrangement. Regarding BETi in clinical development for high-grade B-cell lymphoma or other NHLs, a couple of Phase I trials were completed (NCT04089527) or withdrawn (NCT03925428) in 2022 and 2020, respectively, but no reports have been published yet. Encouragingly, Dickinson *et al.*[54] tested the BETi RO6870810 in combination with venetoclax (a BCL2 inhibitor) and rituximab (an anti-CD20 monoclonal antibody). In this Phase Ib study (NCT03255096), no DHL patients were enrolled, but 10 out of 18 patients (55.6%) were considered DEL. From those, one patient achieved a complete response (CR).

Histone deacetylase inhibitors

Another group of targeted agents that have been under development for even longer (over 30 years) than BETi and represented hope for the treatment of hematologic diseases includes Histone deacetylase (HDAC) inhibitors (HDACi). MYC can recruit epigenetic modifiers, like HDACs, to activate or repress different target genes. Notably, MYC is found acetylated at K423 upon treatment with a pan-HDACi, decreasing MYC transcription due to autoregulation and resulting in apoptosis[55].

Table 1. Summary of the indirect MYC-targeting approaches and their development stage in high-grade B-cell lymphoma

Compound	Combined with other therapies?	Mechanism of action	Clinical or Preclinical	References
CC-95775	No	Reduction of MYC translation via inhibition of the BET family of proteins (BRD4, BRD3, BRD2 and BRDT)	Phase Ib Completed (NCT04089527)	NA
GSK525762C	+ entinostat		Phase I Withdrawn - Protocol moved to disapproved (NCT03925428)	NA
RO6870810	+ venetoclax with or without rituximab		Phase Ib Completed (NCT03255096)	Dickinson *et al.*[54]
Chidamide	+ rituximab	HDAC inhibitors	Preclinical	Guan *et al.*[57]
	+ high-dose rituximab and chemotherapy followed by auto-HSCT		Clinical - Case Report	Kang *et al.*[58]
	+ venetoclax		Preclinical	Luo *et al.*[59]
Marbostat-100	No		Preclinical	Winkler *et al.*[55]
CKD-581	No		Preclinical	Kim *et al.*[61]
			Phase I Completed (NCT01580371)	NA
CUDC-101	+ gemcitabine		Preclinical	Li *et al.*[62]
Compound 8	No		Preclinical	Zhang *et al.*[63]
Tucidinostat	+ R-CHOP		Phase III Recruiting (NCT04231448)	Zhang *et al.*[64]
Fimepinostat (CUDC-907)	No	Dual HDAC and PI3K inhibitor	Phase II Completed (NCT02674750)	Landsburg *et al.*[65]
VIP152	+ immunotherapy	CDK9 inhibitors	Phase I Recruiting (NCT02635672)	Diamond *et al.*[67]
BTX-A51	No		Phase I Recruiting (NCT04872166)	Ball *et al.*[70]
KB-0742	No		Phase I Recruiting (NCT04718675)	NA
SHC014748M	No	PI3K inhibitors	Phase I Unknown (NCT03588598)	Fan *et al.*[80]
WNY1613	No		Preclinical	Zuo *et al.*[81]
CYH33	No		Preclinical	Chen *et al.*[82]
SAF-248	No		Preclinical	Zhang *et al.*[83]
KA2237	No		Phase I Completed (NCT02679196)	Nastoupil *et al.*[84]
BR101801	No		Phase I/II Recruiting (NCT04018248)	Kim *et al.*[86]
Parsaclisib	+ R-CHOP		Phase I/Ib Recruiting (NCT04323956)	Wang *et al.*[88]
TG-1701	No		Preclinical	Ribeiro *et al.*[90]
Ibrutinib	+ CIT (chemoimmunotherapy)		Phase III Terminated (NCT02703272)	Burke *et al.*[91,92]
Zanubrutinib	+ R-CHOP	BTK inhibitors	Phase II Recruiting (NCT05189197)	NA
Acalabrutinib	+ CAR T cell		Phase II Not yet recruiting (NCT05583149)	NA
	+ ACP-319 (PI3Kδi)		Phase I/II Active, not recruiting (NCT02328014)	Barr *et al.*[94]
Pimozide	+ etoposide		Preclinical	Li *et al.*[100]
CS2164 (Chiauranib)	+ venetoclax		Preclinical	Yuan *et al.*[103]
eFT226	+ AKTi/PI3Ki	USP1 inhibitor	Preclinical	Thompson *et al.*[104]
Ixazomib	+ DA-EPOCH-R	Multitarget inhibitor (VEGFR1, VEGFR2, VEGFR3, and c-Kit, CSF-1R, AURKB)	Phase I/II Active, not recruiting (NCT02481310)	Galvez *et al.*[108]

Bortezomib	+ R-CHOP	eIF4A inhibitor	Phase III Completed (NCT01324596)	Davies *et al.*[109,110]
Alisertib	+ romidepsin	Proteasome inhibitors	Phase I Completed (NCT01897012)	Strati *et al.*[111]
MRT-2359	No		Phase I/II Recruiting (NCT05546268)	Gavory *et al.*[112]
		Aurora A kinase inhibitor		
		Degradation of GSTP1 and downregulation of N- and L-MYC		

BTK: Bruton's tyrosine kinase; BET: bromodomain extra-terminal; DA-EPOCH-R: dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, hydroxydaunorubicin, and rituximab; HDAC: Histone deacetylase; R-CHOP: rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone; USP1: ubiquitin-specific protease 1; VEGFR: vascular endothelial growth factor receptor.

Overall, though, the accumulated clinical data with these therapeutics has been somewhat disappointing, as they did not fulfill the promise of the *in vitro* and *in vivo* models. Indeed, only certain hematologic tumors seem to benefit from this type of treatment in the clinical setting, with only five approved drugs, of which four are for T-cell lymphoma and one for multiple myeloma. Interestingly, breast cancer was the first indication, outside hematologic malignancies, to get the approval of an HDAC inhibitor as a combinatorial therapy (tucidinostat plus exemestane, an aromatase inhibitor) in 2019[56].

In the particular case of HGBL, several compounds are under investigation. At the preclinical level, Guan *et al.*[57] identified the promising combination of chidamide (an HDACi approved by the China Food and Drug Administration for T-cell lymphoma) and rituximab (a monoclonal antibody against CD20) as a potential strategy to treat relapsed/refractory (R/R) DLBCL. Indeed, such a combination led to an impressive reduction of tumor volume in a DLBCL cell line-derived xenograft mouse model. The same authors also report the clinical case of a patient that achieved partial response (PR) after only one cycle and underwent CR after three cycles of treatment[57]. Similarly, Kang *et al.*[58] studied the effect of a modified conditioning regimen with chidamide and high-dose rituximab for THL, followed by autologous hematopoietic stem cell transplantation (auto-HSCT), showing 2 CRs, while a third patient, who was insensitive to the chemotherapy, did not respond.

In a different study, Luo *et al.*[59] proposed the combination of chidamide with venetoclax for the treatment of DHL. They demonstrated a synergistic effect of the compounds both *in vitro* and *in vivo* using SU-DHL-4 (MYC/BCL2 DEL) and DB (*MYC*/*BCL2* rearranged) cell lines. Importantly, chidamide caused a reduction in MYC levels in both cell lines, suggesting that suppression of MYC is achieved regardless of the rearrangement status[59].

In contrast, the research by Winkler *et al.*[55] described a new molecule, Marbostat-100 (M-100), an HDAC6 inhibitor that targets almost exclusively lymphoma cells with high MYC levels. Contrary to previous observations with the pan-HDACi MS-275, M-100 caused MYC reduction by proteasomal degradation, while *MYC* transcription was not affected, and extended survival of transgenic Eμ-*myc* mice *in vivo*. Importantly, the authors underline that MYC needs to be Threonine58 (T58) wild type for its degradation to happen efficiently[55], as a mutation of this residue on the MYC protein prevents a phosphorylation event critical to its proficient proteasomal degradation[60]. In fact, Cucco *et al.*[40] identified a high frequency of *MYC* T58A and P57S mutations in the MHG subset of DLBCL.

Kim *et al.*[61] assessed the efficacy of CKD-581, another HDACi that was previously tested in a Phase I clinical trial (NCT01580371), but for which no safety and pharmacokinetic results were published. Preclinically, this broad-spectrum HDACi decreased the expression of both MYC and BCL2 and was able to elicit a significant reduction in tumor growth. Similarly, other recent preclinical studies showed the potential of HDACi for the treatment of some types of NHL[62,63].

More advanced in their development, tucidinostat and fimepinostat (CUDC-907) have been or are being evaluated in Phase II/III trials (NCT02909777, NCT02674750). Zhang *et al.*[64] showed improved 2-year progression-free survival (PFS) and overall survival (OS) in DEL patients, as well as a lessened negative prognostic impact of *CREBBP*/*EP300* (*Histone acetyltransferase p300*) mutations when treated with tucidinostat in combination with R-CHOP. Furthermore, fimepinostat is a dual HDAC and PI3K inhibitor reported to have an objective response rate (ORR) of 15% in patients with relapsed/refractory DLBCL or HGBL with high MYC (≥ 40%). ORR was further increased to 22% when the patients were classified based on a 3-protein biomarker. Such a discovery led the authors to suggest that combinatorial therapies or biomarker-assisted stratification could improve even more the response to treatment[65].

Cyclin-dependent kinase 7/9 inhibitors

Cyclin-dependent kinase (CDK) inhibitors have been the topic of intense research for two decades. In particular, CDK9 emerged as a druggable target for the development of cancer therapeutics, due to its crucial role in the transcriptional regulation of both short-lived anti-apoptotic proteins and oncogenes, such as *BCL2/6* and *MYC*, critical for the survival of transformed cells[66].

VIP152 is a potent and highly selective CDK9 inhibitor that, in a Phase I study, led to complete metabolic remission by PET-CT in two out of seven patients with HGBL[67], and stabilization of the disease in seven out of 30 patients with non-lymphoma solid tumors[67,68]. Preclinical models using the SU-DHL-10 HGBL cell line in a murine xenograft recapitulated the remission observed clinically in a dose-dependent manner. Additionally, pharmacodynamic biomarker analysis *in vitro* demonstrated a transient downregulation of the mRNA of MYC, MCL-1 and Proliferating Cell Nuclear Antigen (PCNA), resulting in a durable clearance of these oncogenic drivers[69]. Frigault *et al.*[69] also reported a transient biomarker modulation in 7 stable HGBL patients of their study when evaluating short-lived transcripts by RNAseq from whole blood samples. In particular, they observed a 70% reduction in MYC, MCL1 and PCNA mRNA post-dose.

Ball *et al.*[70] presented at the 2022 ASCO annual meeting the interim results of the first-in-human trial of BTX-A51 (NCT04872166), a direct inhibitor of casein kinase 1α (CK1α), CDK7 and CDK9 that robustly increases p53 protein levels via CK1α inhibition, while preferentially decreasing super-enhancer transcription of MYC and MCL-1. In the second part of the study, they are planning to enroll up to 40 additional subjects to evaluate the safety and preliminary efficacy in patients with documented MYC genomically amplified or overexpressed tumors, such as HGBL (NHL)[71]. BTX-A51 in monotherapy has been found to have an acceptable safety profile and promising antileukemic activity[71].

Another interesting orally bioavailable CDK9i is KB-0742, which demonstrated preclinical efficacy in AR-dependent castration-resistant prostate cancer[72] and transcriptionally addicted tumors, such as sarcoma and chordoma[73]. It is currently being tested in a Phase I clinical trial that includes DLBCL with MYC translocation and Burkitt lymphoma (NCT04718675).

Phosphoinositide 3-kinases inhibitors

PI3K/AKT/mTOR (PAM) signaling is involved in important physiological and pathophysiological functions that drive tumor progression, such as metabolism, cell growth, proliferation, angiogenesis and metastasis[74]. In particular, different lymphoma subtypes (a subset of DLBCL and BL) have been shown to rely on BCR survival signals mediated by the phosphoinositide 3-kinases (PI3K) pathway to promote the proliferation and survival of malignant B-cells[75-77]. One of the main downstream targets of the PAM pathway is MYC: phosphorylation at T58 occurs via glycogen synthase kinase 3 (GSK3B), which is a direct target of AKT[78]. Moreover, activation of mTOR and AKT activity directly increases the translation of MYC[78] and activation of PAM leads to the phosphorylation and degradation of Mad1, the natural antagonist of MYC[79]. Pharmacological suppression of this pathway has been proposed as an anti-neoplastic approach. Small-molecule inhibitors targeting PI3K consist of pan-, isoform-specific and dual PI3K/mTOR (Mammalian Target of Rapamycin) inhibitors.

Several groups in China have identified new PI3Ki and tested them against panels of B-cell lymphoma cell lines, including DLBCL and some examples of HGBL, such as DOHH2, SU-DHL-6 and SU-DHL-10:

Fan *et al.*[80] showed *in vitro* activity of their compound SHC014748M, a PI3Kδ inhibitor, which also caused a significant reduction in tumor volume in a xenograft mouse model with the SU-DHL-6 cell line. Remarkably, SHC014748M performed better than idelalisib, an FDA-approved drug for the treatment of NHL. Currently, SHC014748M is being evaluated against indolent relapsed/refractory B-cell NHL, including chronic lymphocytic leukemia (CLL), FL, marginal zone lymphoma (MZL) and Waldenstrom's macroglobulinemia (WM), in a Phase I clinical trial (NCT03588598).

Similarly, Zuo *et al.*[81] found their candidate WNY1613, also a PI3Kδ inhibitor, to exert antiproliferative effects *in vitro* in a panel of NHL cell lines (seven DLBCL, two mantle cell lymphoma (MCL) and one BL). They also demonstrated that their inhibitor can prevent tumor growth in two mouse xenograft models of SU-DHL-6 and JEKO-1 cell lines. The pharmacodynamics and pharmacokinetics of this molecule are under current investigation.

In independent studies, Chen *et al.*[82] (using CYH33 against PI3Kα) and Zhang *et al.*[83] (using SAF-248 against PI3Kδ) evidenced that both compounds were effective in blocking B-cell lymphoma cell growth *in vitro* and *in vivo*, partly by causing the downregulation of MYC. As happened with SHC014748M, SAF-248 displayed superior activity compared to idelalisib, which is also reported to be far more toxic. Importantly, Zhang *et al.* demonstrated that the expression of PI3Kα was negatively correlated with the activity of SAF-248 and found multiple oncogenic pathways, such as IL2_STAT5 (Interleukin-2/Signal Transducer and Activator of Transcription 5), IL6_STAT3 (Interleukin-6/Signal Transducer and Activator of Transcription 3), MTORC1 (Mammalian Target Of Rapamycin Complex 1) and MYC, upregulated in tumor tissues upon prolonged treatment with SAF-248 compared to those with short-term administration, possibly pinpointing to an adaptive mechanism of resistance[83]. In line with this observation, Chen *et al.*[82] proposed the combination with BETi or HDACi to overcome the adaptive resistance to PI3K inhibition, which could be attributed to increased H3K27Ac and binding of CREB Binding Protein (CBP)/p300 with BRD4 proteins gene loci of a subset of growth factors and receptors.

Some other PI3Ki under clinical development for the treatment of B-cell lymphoma include KA2237, BR101801 and parsaclisib. Nastoupil *et al.*[84] reported KA2237, a dual PI3K β/δ inhibitor, to have a manageable toxicity profile and to be an effective therapeutic option for patients with refractory B-cell lymphoma without an acceptable standard of care option. In a Phase I clinical study with the compound, 19

out of 21 patients were evaluable for response, and 8 of the evaluable ones had DLBCL. Two of the DLBCL patients achieved CRs and another one achieved a PR[84].

BR101801, a triple inhibitor of PI3K γ/δ and DNA-PK, showed highly potent effects in blocking cellular proliferation of NHL cell lines in preclinical models, including both the indolent and aggressive subtypes[85]. Clinically, the compound was well tolerated and showed preliminary signs of activity in patients with relapsed/refractory hematologic malignancies. The Phase Ib/II study of BR101801 is warranted in relapsed/refractory NHL[86].

Finally, parsaclisib, the most advanced PI3Ki in clinical development described here, inhibits PI3K δ. Shin *et al.*[87] found that overexpression of MYC turned some of the DLBCL cell lines insensitive to parsaclisib treatment. The resistance could be overcome by the addition of a BETi that reduced MYC levels. Recently, Wang *et al.*[88] investigated the feasibility of combining parsaclisib with standard immunochemotherapy (R-CHOP), seeking signs of efficacy. They chose the population of study to be high-risk lymphoma patients bearing MYC rearrangements or translocations (i.e., HGBL) or overexpression of MYC or BCL2. In the interim report, 13 patients were evaluable, 8 achieved CR, 4 PR and one progressed[88]. Given the encouraging preliminary efficacy, parsaclisib plus R-CHOP could constitute an experimental arm in future frontline DLBCL trials investigating genetic subtype-driven novel therapies.

Bruton's tyrosine kinase inhibitors

Ever since the discovery of the involvement of tyrosine kinases (TK) in cancer, which led to their consideration as valuable targets for cancer treatment, a broad spectrum of TKis has been launched, including Bruton's tyrosine kinase inhibitors (BTKis). Previous genomic studies suggested that some components of the B-cell receptor (BCR) signaling pathway are MYC transcriptional targets. Moreover, it has been demonstrated that, in pre-malignant B cells from the Eμ-*myc* mouse model, MYC overexpression is sufficient to activate BCR and PI3K/AKT signaling pathways, while conferring resistance to pharmacologic inhibitors of the BCR signaling pathway, like BTKis[89].

For the treatment of B-NHL, some preclinical studies with TG-1701 showed improved efficacy compared to ibrutinib, the first BTKi approved by the FDA, and described MYC downregulation, both at the mRNA and protein level, as part of the signature observed in early-responder patients, as well as in BTKi-sensitive B-NHL cell lines and xenografts[90].

In this sense, Burke *et al.*[91] reported promising preliminary efficacy findings in a Phase III clinical trial with pediatric patients with R/R mature B-NHL, who have a poor prognosis, treated with ibrutinib in combination with rituximab, ifosfamide, carboplatin and etoposide (RICE) modified with dexamethasone or with rituximab, vincristine, ifosfamide, carboplatin, idarubicin and dexamethasone (RVICI). More than half of the patients (12/21) responded to the therapies, achieving a total of 5 CR and 7 PR. However, as the final endpoint was event-free survival (EFS), the study was stopped early for futility, given that ibrutinib did not improve EFS in this population in combination with chemotherapy backbones. The authors suggest prioritizing, instead, bispecific antibodies, antibody-drug conjugates and CAR-T cells[92].

Even though BTKis have shown modest therapeutic activity in DLBCL (i.e., ORR of 23% for ibrutinib in relapsed patients, of 24% for acalabrutinib in DLBCL patients regardless of their molecular subtype, and of 36% for zanubrutinib in ABC DLBCL patients), Yang *et al.*[93] performed retrospective biomarker assessments and showed that zanubrutinib could have antitumor activity in patients with mutations in Cluster of Differentiation 79-B (CD79B) and Myeloid Differentiation primary response 88 (MYD88).

Hence, they propose future studies to focus on developing mechanism-based treatment combinations and biomarker-driven patient selection. In line with this reflection, Fudan University is evaluating zanubrutinib in combination with the immunochemotherapy R-CHOP in patients with non-GCB DLBCL with co-expression of MYC/BCL2, based on the findings of a posthoc analysis on four studies, in which an ORR of 61% and PFS of 5.4 months was achieved in patients with MYC and BCL2 overexpression treated with zanubrutinib (NCT05189197).

Acalabrutinib, as zanubrutinib, is another potent selective, irreversible BTKi with minimal off-target effects, being evaluated in combination with CAR-T cell therapy or with PI3Ki in Phase II clinical trials (NCT04257578). Barr *et al.*[94] investigated the combination of acalabrutinib with ACP-319 in relapsed/refractory B-cell NHL. They showed in patients with non-GCB DLBCL an ORR of 63% (10 out of 16 patients; CR rate 25%) with a median duration of response of 8.2 months. Of the ten responders, six were double expressors, overexpressing MYC and BCL2/BCL6. No responses were observed in the nine patients with GCB DLBCL. Another Phase II study should start soon to evaluate the effectiveness and safety of acalabrutinib combined with lisocabtagene maraleucel (liso-cel) in relapsed/refractory aggressive B-cell lymphoma patients (NCT05583149).

MYC/MAX antagonists

While all the approaches listed above have targeted MYC indirectly, there is also the possibility of attacking it directly. Perhaps the most common strategy in this context focuses on the disruption of the interaction of MYC with its natural partner MAX. In this case, most data in the literature are related to the use of small molecules (SM), although MYC lacks significant secondary and tertiary structure when not complexed with other proteins, making specific recognition by SM quite difficult[95]. The first one to show effect in lymphoma was 10058-F4, which was employed *in vitro* in different BL cell lines and demonstrated that targeting of MYC/MAX interaction could impair lymphoma growth in a time- and dose-dependent manner[96]. Later on, 7-nitro-*N*-(2-phenylphenyl)-2,1,3-benzoxadiazol-4-amine (10074-G5) was used in Daudi BL cells, where, again, it inhibited cell growth *in vitro*, but failed to affect growth in xenografts in C.B-17 SCID mice, likely due to poor bioavailability[97]. Another SM, sAJM589, was more recently identified in a Principal Component Analysis (PCA)-based high-throughput screen. sAJM589 was able to inhibit the transcription of MYC target genes in P493-6 BL cells, as well as to suppress the proliferation of diverse MYC-dependent cancer cell lines and anchorage-independent growth of Raji cells[98]. However, the most advanced MYC/MAX dimerization inhibitor in clinical development is Omomyc, a MYC dominant negative based on the basic helix-loop-helix leucine zipper (b-HLH-Z) domain of the human c-MYC protein, able to form homodimers and heterodimers with MYC and MAX and interfere with MYC transcriptional activity. Omomyc has been used in its transgenic form in BL, in murine lymphoma cell lines obtained from Eμ-*myc* transgenic mice and in Raji cells (wild-type or knock out for FBXO11) *in vitro* and *in vivo* (subcutaneous xenografts), alone or in combination with the BCL6 degrader BI-3802[99]. In this context, Omomyc blocked cell proliferation and increased apoptosis, effects further improved by combined BCL6 targeting. Remarkably, the first Omomyc-derived drug product, OMO-103, has just successfully completed a Phase I clinical trial (NCT04808362) in all-comers solid tumors. However, its safety and efficacy in hematologic diseases have not been tested in the clinical setting yet. Hopefully, this will soon be the case.

Other inhibitors and degraders

Further preclinical or clinical studies are assessing the efficacy of novel targets or repurposed molecules for the treatment of MYC-overexpressing lymphoma.

Li *et al.*[100] elucidated the role of ubiquitin-specific protease 1 (USP1) in B-cell lymphoma. USP1 is highly expressed in DLBCL patients and was found to be associated with poorer prognosis. Using pimozide, a USP1 inhibitor, the authors could stop DLBCL cells from cycling. Mechanistically, they found USP1 to stabilize MAX, promoting the transcription of MYC target genes. Importantly, when combined with etoposide, a chemotherapeutic, pimozide blocked the tumor growth in a xenograft mouse model resistant to immunochemotherapy R-CHOP. Since pimozide is already approved by the FDA for the treatment of other diseases and is known to cause low toxicity, the authors propose the combination as an attractive alternative for DLBCL patients resistant to R-CHOP.

On a different note, CS2164 is an orally bioavailable multitargeted inhibitor being evaluated in numerous clinical studies with promising clinical anti-cancer effects and tolerable toxicities[101]. Deng *et al.* observed a potential role of CS2164 for the treatment of NHLs through the perturbation of multiple signaling cascades (angiogenesis, inflammation and proliferation) by inhibiting the following kinases: vascular endothelial growth factor receptor 1-3 (VEGFR1, VEGFR2 and VEGFR3), platelet-derived growth factor receptor alpha (PDGFRα), receptor tyrosine kinase (c-Kit), kinase Aurora B (AURKB), and chronic inflammation-related kinase (CSF-1R). Specifically, CS2164 showed superior anti-lymphoma activity against MYC-arranged BL models, suggesting it could also have a cytotoxic effect on other MYC-altered malignancies[102]. Following these observations, Yuan *et al.*[103] assessed the efficacy of this novel agent in combination with venetoclax using HGBL *in vitro* and *in vivo* models, where they could demonstrate a reduction of MYC and BCL2 protein levels, as well as antitumor efficacy with tolerable toxicities in a xenograft mouse model with MCA cells.

Interestingly, Thompson *et al.* found that activation of the PI3K/mTOR signaling pathway results in the activation of eukaryotic initiation factor-4A (eIF4A), required for the translation of oncoproteins, like MYC. The authors used eFT226 to inhibit the translation of specific mRNAs by promoting eIF4A1 binding to 5′-untranslated regions (UTR) containing polypurine and/or G-quadruplex recognition motifs[104]. The compound blocked the proliferation of GCB-DLBCL tumor models Pfeiffer and SU-DHL6. When assessing the pharmacodynamic response to the drug, the authors reported downregulation of MYC, Cyclin D1, and BCL6 protein levels in a time- and exposure-dependent manner. Collectively, their results support the clinical development of eFT226 in patients with B-cell malignancies.

A different class of molecules that emerged as an important therapeutic strategy in hematologic malignancies, particularly in multiple myeloma, are proteasome inhibitors[105]. Ravi *et al.*[106] demonstrated that ixazomib sensitivity is mediated through checkpoint kinase 1 (CHK1)-dependent MYC function involving histone acetylation, and demonstrated that dual inhibition of CHK1 and MYC induced synergistic cell death with ixazomib. Based on preclinical data showing MYC downregulation, a couple of clinical studies were started to evaluate the efficacy of ixazomib in lymphoma patients with different subtypes (DLBCL, FL, MZL, MCL, transformed FL, DLBCL-FL, and some other indolent forms) in combination with immune- or immunochemotherapy. Graf *et al.*[107] concluded that once-weekly oral ixazomib showed a favorable safety profile and considerable activity in the frontline treatment of indolent B-cell NHL, with the best results in FL. When combined with a single 4-week course of rituximab, ixazomib achieved durable disease control with very low toxicity in the majority of patients with FL. On the other hand, Galvez *et al.* reported DA-EPOCH-R induction with adjunctive ixazomib followed by maintenance ixazomib to be safe and effective in an older population of HGBL. The ORR after induction was 89%, with an associated CR rate of 61%[108].

Moreover, Sha *et al.*[39] reported that the addition of bortezomib to the R-CHOP regimen (RB-CHOP) could be beneficial for the MHG subgroup in a randomized Phase III study (NCT01324596). Indeed, Davies *et al.* presented at the ASH 2022 Annual Meeting extended data on the REMoDL-B trial, where 5-year PFS was significantly improved for MHG patients (PFS 29% with R-CHOP *vs.* 55% with RB-CHOP)[109,110].

Lastly, Strati *et al.*[111] recently (2020) discussed the results of their Phase I trial assessing the combination of alisertib (an Aurora A kinase inhibitor) with romidepsin (and HDACi) for the treatment of patients with relapsed/refractory B- and T-cell lymphomas (NCT01897012). Unfortunately, the cytokinesis failure observed in preclinical models when combining both agents only happened in T-cells in the clinical setting. Hence, significant myelosuppression and limited ORR (< 20%) for B-cell NHL patients do not support further clinical evaluation of alisertib.

On a more positive note, novel promising agents, such as MRT-2359, a molecular glue degrader directed against GSTP1 developed by Monte Rosa Therapeutics, are under development. Preclinical results on MRT-2359 were presented last year at the AACR Annual Meeting and the 34th EORTC-NCI-AACR Symposium, with the promise of being effective in MYC-driven tumors addicted to protein translation[112]. MRT-2359 is being evaluated in a Phase I/II trial that started recruitment very recently (NCT05546268)

CONCLUSION

Assessment of MYC rearrangements or expression is typically used in lymphomas and other hematologic malignancies as a diagnostic tool[113,114]. These cancers have gained the classification of MYC-associated tumors, because this oncoprotein has been demonstrated to play a key role in the physiopathology of these diseases[115,116]. In fact, according to the concept of oncogene addiction, multiple studies have shown that MYC inactivation causes tumor regression in the context of lymphomas[41-44]. In this review, we summarized the approaches explored so far to target MYC-dependency, *in vitro*, *in vivo* and in the clinical setting. However, it is important to stress that most of these approaches have been so far indirect ones and there is no set of experiments that established to which extent different cancer cells are dependent on MYC activity or, in other words, how much MYC inhibition is required to stop their proliferation or cause cell death.

Based on the above, several questions remain still unanswered: (i) would direct MYC inhibition be a more effective therapeutic strategy than indirect targeting of it in the context of lymphoma? (ii) are MYC levels a good predictive biomarker of response to MYC inhibition in every context? (iii) what is the ideal way of assessing MYC function? Would mRNA expression, protein expression, and determination of gene amplifications or rearrangements be sufficient? (iv) is MYC activity a biomarker that can potentially stratify good and bad responders to therapies in general? (v) is it possible to define a unique MYC transcriptional signature shared across various tumor types?

Our knowledge continues to expand as more efforts are dedicated to designing viable therapies that target MYC. Nevertheless, we believe it is critical answering some of the questions above to improve our understanding of MYC biology and develop the best MYC-targeting approaches.

DECLARATIONS

Authors' contributions

Conceptualization, investigation, writing: Martínez-Martín S, Beaulieu ME
Conceptualization, supervision, writing: Soucek L

Availability of data and materials

Not applicable.

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Conflicts of interest

Soucek L and Beaulieu ME are co-founders and shareholders of Peptomyc S.L. and inventors of patent application WO2014180889 A8 that covers the use of the Omomyc mini-protein in medicine, held by VHIO and licensed to Peptomyc. Martínez-Martín S is an employee of Peptomyc S.L.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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How acute myeloid leukemia (AML) escapes from FMS-related tyrosine kinase 3 (FLT3) inhibitors? Still an overrated complication?

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Abstract

FMS-related tyrosine kinase 3 (FLT3) mutations, present in about 25%-30% of acute myeloid leukemia (AML) patients, constitute one of the most frequently detected mutations in these patients. The binding of FLT3L to FLT3 activates the phosphatidylinositol 3-kinase (PI3K) and RAS pathways, producing increased cell proliferation and the inhibition of apoptosis. Two types of FLT3 mutations exist: FLT3-ITD and FLT3-TKD (point mutations in D835 and I836 or deletion of codon I836). A class of drugs, tyrosine-kinase inhibitors (TKI), targeting mutated FLT3, is already available with 1st and 2nd generation molecules, but only midostaurin and gilteritinib are currently approved. However, the emergence of resistance or the selection of clones not responding to FLT3 inhibitors has become an important clinical dilemma, as the duration of clinical responses is generally limited to a few months. This review analyzes the insights into mechanisms of resistance to TKI and poses a particular view on the clinical relevance of this phenomenon. Has resistance been overlooked? Indeed, FLT3 inhibitors have significantly contributed to reducing the negative impact of FLT3 mutations on the prognosis of AML patients who are no longer considered at high risk by the European LeukemiaNet (ELN) 2022. Finally, several ongoing efforts to overcome resistance to FLT3-inhibitors will be presented: new generation FLT3 inhibitors in monotherapy or combined with standard chemotherapy, hypomethylating drugs, or IDH1/2 inhibitors, Bcl2 inhibitors; novel anti-human FLT3 monoclonal

antibodies (e.g., FLT3/CD3 bispecific antibodies); FLT3-CAR T-cells; CDK4/6 kinase inhibitor (e.g., palbociclib).

Keywords: Acute myeloid Leukemia, FLT3, gilteritinib, midostaurin, TKI-inhibitor resistance, quizartinib, sorafenib, crenolanib

INTRODUCTION

In 1996, the first unexpectedly longer transcripts of FMS-like tyrosine kinase 3 (FLT3) were reported in the transmembrane domain through the juxtamembrane domain of the gene[1]. This represented a cornerstone discovery in acute myeloid leukemia (AML), since internal tandem duplications (ITD)-FLT3 mutations represent one of the most recurrent alterations, occurring in 22-32% and the remaining 8% including the tyrosine kinase domain (TKD), *de novo* AML[2,3]. Moreover, FLT3-ITD mutations, particularly those carrying a high FLT3-ITD mutant to wild-type allelic ratio when detected at initial diagnosis, seem to confer a short duration of remission and globally a worse prognosis[4]. The presence of FLT3-ITD appears to be strongly associated with hyperleukocytosis and high blast percentages in both peripheral blood and bone marrow at presentation[5]. From a morphologic standpoint, it has been suggested that AML with cup-like nuclei is associated with co-occurring mutations of both NPM1 and ITD- or TKD- FLT3[6]. Most importantly, we have an interesting class of drugs targeting FLT3, tyrosine-kinase inhibitors (TKI), which have an already long history in the treatment of this cancer. With the availability of 1st and 2nd generation inhibitors, the emergence of resistance or the selection of clones not responding to FLT3 inhibitors has become an important clinical dilemma, probably recalling the challenge of TKI in CML and ALL *BCR*: *ABL* positive. This review will try to summarize the evidence, from a clinical viewpoint, regarding the development of resistance to FLT3-inhibitors, their real clinical impact in AML patients, and strategies to obviate the development of resistance.

FLT3 GENE AND ITS MOLECULAR FUNCTION

FLT3 (Fms-like tyrosine kinase 3) has strong similarities in its sequence with other members of the class III receptor tyrosine kinase (RTKIII) receptor family (FMS, platelet-derived growth factor receptor (PDGFR) α and β, and KIT)[7]. FLT3 is composed of an extracellular domain consisting of 5 immunoglobulin-like (Ig-like) domains, and by a cytoplasmic domain with a juxtamembrane domain and two intracellular tyrosine-kinase domains (TKDs)[8]. Physiologically, FLT3 is expressed over the cytoplasmic membrane of immature myeloid and lymphoid progenitor cells[9] and is activated by its ligand FLT3L, which can be found in the cytoplasm of bone marrow stromal fibroblasts, T-cells, B-cells, and progenitors CD34+ [Figure 1][10]. Noteworthy, FLT3 (CD135) is expressed on the surface of more than half of AML and seems associated with a worse outcome[11]. The binding of FLT3L to FLT3 activates the phosphatidylinositol 3-kinase (PI3K) and RAS pathways, producing increased proliferation and impaired apoptosis in cells. Moreover, activated PI3K stimulates downstream proteins like 3-phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (PKB/AKT) and the mammalian target of rapamycin (mTOR), and PI3K activation hampers apoptosis through phosphorylation of the pro-apoptotic protein BAD, member of the BCL2-family. Moreover, the activation of RAS stimulates other downstream effectors like RAF, MAPK/ERK kinases (MEKs), extracellular-signal-regulated kinase (ERK), and the 90-kDa ribosomal protein S6 kinase (RSK). These downstream effectors can activate signal transducers and activators of transcription (STATs), which lead to the transcription of genes involved in cellular proliferation[7]. On the other hand, activating genetic mutations of FLT3 can produce the abnormal expression of a constitutively activated tyrosine kinase receptor in AML blasts, which is independent of the physiological FLT3L stimulus[12].

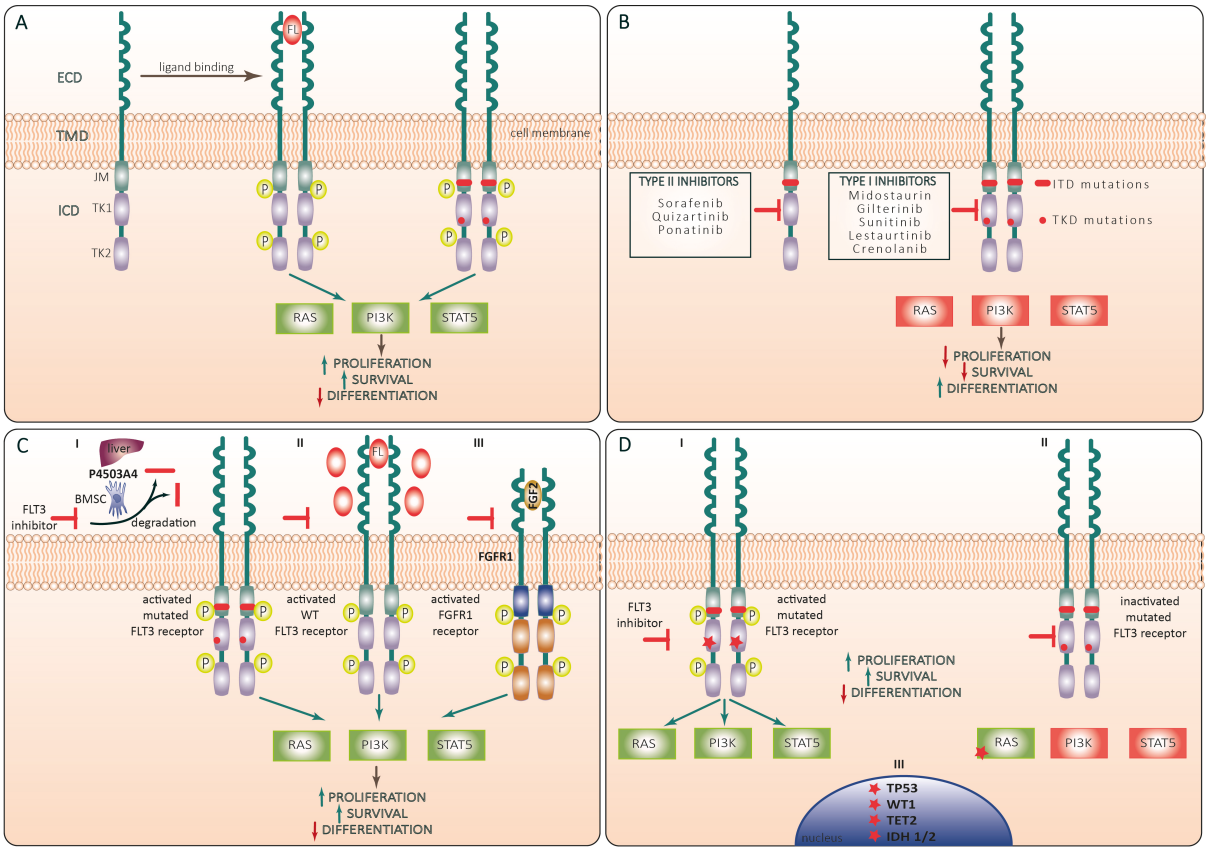

Figure 1. (A) Structure and activation of FLT3 receptor. FLT3 molecule is a tyrosine kinase receptor composed of an extracellular domain (ECD) that comprises 5 immunoglobuline-like repeats, a short transmembrane domain (TMD) and an intracellular domain (ICD) that includes a juxtramembrane (JM) domain followed by two tyrosine kinase (TK) domains (TK1 and TK2). Binding of the FLT3 ligand (FL) to ECD leads to formation of a ternary complex between FL and two receptor subunits with consequent conformational changes and trans-phosphorylation of TK and JM domains. The activated receptor exerts a tyrosine kinase activity against a series of adaptor and effector molecules leading to activation of Ras/Raf/MAPK (RAS) PI3K/Akt (PI3K) and Jak/STAT5 (STAT5) pathways promoting leukemic cell proliferation and survival and suppressing differentiation. Both FLT3-ITD and FLT3-TKD mutations lead to ligand-independent activation of FLT3 signaling; (B) types of FLT3 inhibitors. Type I FLT3 inhibitors interact with the ATP-binding site of the intracellular TK domain (TKD) when the receptor is in active conformation, whereas Type II inhibitors bind to the inactive form of the receptor, thus preventing its activation. Type I inhibitors inhibit FLT3 signaling in AML cells harboring ITD or TKD mutations, whereas Type II inhibitors are active against AML with ITD but not TKD mutations; (C) mechanisms of primary resistance to FLT3 inhibitors: Increased proliferation and survival and decreased differentiation of AML cells may be sustained through (I) Cytochrome P450 3A4-mediated degradation of FLT3-inhibitors by hepatocytes (liver symbol) and BM stromal cells (BMSC); (II) Restoration of FL/FLT3 signaling through compensatory overexpression of wild-type (WT) FLT3 receptor and FL; (III) Activation of common FLT3 and fibroblast growth factor receptor type 1 (FGFR1) downstream target pathways through the increased fibroblast growth factor 2 (FGF2)/FGFR1 signaling; (D) mechanisms of secondary resistance to FLT3 inhibitors: (I) Secondary resistance of FLT3 receptor to pharmacological inhibition may be conferred through newly acquired activating mutations of FLT3 TKD (red asterisk), and upregulation of pro-survival mechanisms through acquired activating mutations in RAS/MAPK pathway genes (II) and epigenetic modifiers like DNMT3A, TET2, IDH2 or transcriptional regulators like WT1 and TP53 (III). Green and red rectangles correspond to activated and inactivated FLT3 downstream signaling pathways, respectively. Yellow "P" circles indicate phosphorylated receptor domains. Green and red arrows indicate positive and negative regulation, respectively.

Two kinds of FLT3 mutations have been individuated: FLT3-ITD and FLT3-TKD (including point mutations in D835 and I836 or deletion of codon I836)[13]. FLT3-ITD takes place between exons 14 and 15. When AML cells carry FLT3-ITD, PCR products generate a wild-type band and a larger ITD band after electrophoresis. D835 and I836 codons are encoded by the nucleotide GATATC, which forms the Eco-RV restriction site. The amplified products of wild-type FLT3 are digested into two bands by the Eco-RV enzyme, while amplified products with D835-mutations (FLT3-TKD) result in uncut bands, and this

permits a traditional diagnosis[14]. Nowadays, several methods have been developed or adapted to help identify mutations of FLT3 and aberrant karyotypes (e.g., Multiplex-targeted next-generation sequencing)[15], and moreover, next-generation sequencing (NGS) techniques for Measurable residual disease (MRD) detection are under development for clinical practice[16]. Especially in the pre-FLT3 inhibitors era, patients harboring FLT3-ITD had a worse prognosis in terms of OS and relapse-free survival[17]. Moreover, some studies suggested that a high Mutant-to-wild-type allelic ratio of FLT3-ITD was associated with a worse prognosis[4,18]. Other studies, however, found no increased risk of relapse in patients with FLT3-ITDhigh[19]. Other important determinants of prognosis in patients with FLT3-ITD are the karyotype status[20] and NPM1mut which confers a more favorable outcome[21,22]. Indeed, patients harboring NPM1mut and FLT3-ITDhigh are classified as intermediate risk in ELN2017[23]. Different is the case for AML with FLT3-TKD; for these patients, a clear impact on prognosis is not established due to conflicting results[24-30]. However, in the new ELN2022 classification, probably due to the therapeutic benefit of FLT3 inhibitors in the new AML scenario, all patients with FLT3 mutation are classified as intermediate risk regardless of whether the mutation is detected alone or with other co-mutations. However, after the introduction of FLT3-inhibitors, this prognostic disadvantage of FLT3-mutations seems abrogated, improving levels of MRD[31,32]. MRD techniques to monitor FLT3 mutations are still being explored. In the next future, the improvement and the standardization of next-generation sequencing analysis could strongly increase and refine the opportunities for monitoring FLT3 mutations during treatment.

CLASS OF DRUGS: FLT3-INHIBITORS

The class of drugs of FLT3-inhibitors can be categorized according to chronological order in 1st generation (sorafenib and midostaurin) and 2nd generation (gilteritinib, quizartinib, crenolanib). Another classification is according to the capacity of the TKI to link the active and inactive status of the mutated FLT3 (Type 1: midostaurin, gilteritinib) or the inactivated only status of FLT3 (Type 2: quizartinib, sorafenib)[33]. An important clinical implication is that Type 2 inhibitors at therapeutic concentrations are unable to inhibit FLT3-TKD, and, especially for the D835 mutation, this favors the active conformation of FLT3 and alters the binding of TKIs[34].

Midostaurin is a first-generation TKI of class 1 and was the first drug to be approved for clinical use in FLT3-mutated AML in 2017[35]. Midostaurin has moderate activity as a single agent, as shown in 92 patients who achieved a reduction of bone marrow blasts in 71% of cases[36]. This evidence paved the way for a phase III trial of the association of 7 + 3 and midostaurin. From 2008 to 2011, the RATIFY trial enrolled a total of 717 patients harboring a mutated FLT3 (22.6% had the TKD mutation). The 4-year overall survival (OS) rate was 51.4% in the midostaurin group and 44.3% in the placebo group and the Median event-free survival (EFS) was 8.2 months in the midostaurin group *vs.* 3.0 months[31]. In a post-hoc analysis in patients with FLT3-TKD, the 5-year EFS rate was significantly extended in patients treated with midostaurin than in the placebo arm (45.2% *vs.* 30.1%; $P = 0.044$)[37].

Gilteritinib, which belongs to the 2nd generation of TKI, has more profound single-agent activity than 1st generation drugs and has activity against both FLT3-ITD and TKD mutations[38]. Indeed, in phase I/II study with gilteritinib, 40% of patients achieved a response, with 19 (8%) reaching complete remission[39]. This fueled interest in a randomized phase 3 trial comparing gilteritinib against any chemotherapy (selected by investigators) in relapsed/refractory (R/R) AML with mutated FLT3. In the ADMIRAL trial, the median OS was significantly longer in patients treated with gilteritinib than among those receiving chemotherapy (9.3 *vs.* 5.6 months). The Overall response to gilteritinib was 67.6% and 25.8% for the chemotherapy arm[17]. These favorable results led to the approval of gilteritinib in the relapsed setting[40]. Interestingly, with a prolonged follow-up at 2 years after the first analysis, in the gilteritinib arm, the median duration of CR was

23 months and 16 patients remained on gilteritinib for a prolonged time[41]. Therefore, even if the majority of responding patients also underwent HSCT, there is a subgroup of patients that achieved a prolonged response to gilteritinib.

Sorafenib, a multitargeted TKI, is approved for hepatocellular carcinoma and renal cell carcinoma, but its off-label use in AML, especially after HSCT, is performed in many centers. Sorafenib is active only in FLT3-ITD AML with mild single-agent activity in the relapsed setting[42,43]. After HSCT, a synergism between sorafenib and allogeneic immunity after HSCT has been hypothesized[44], even if the data are conflicting[45]. In a phase II study SORMAIN trial, 43 patients were randomized to receive a prophylactic treatment with sorafenib after HSCT and 40 patients to receive placebo. The 24-month RFS probability was 85% with sorafenib *vs.* 53.3% with placebo (P = 0.002)[46]. Moreover, different phase II studies explored the addition of sorafenib to standard induction chemotherapy, but failed to improve EFS and survival at the cost of increased toxicity[47-49].

Quizartinib, a second-generation TKI, was specifically selected from a library of drugs to deeply inhibit FLT3-ITD, while it is inactive against FLT3-TKD[50]. It has clinical activity as a single agent in the relapsed setting, where 56% of FLT3-ITD-positive patients achieved a composite CR[51]. However, the FDA expressed concerns about the lack of improvement in EFS, so quizartinib was initially rejected. Recently, the FDA granted quizartinib Priority Review for newly diagnosed (ND) FLT3-ITD+ AML based on data from the QuANTUM-First study. QuANTUM-First was a pivotal trial randomizing quizartinib *vs.* placebo added to standard 7 + 3 backbone in FLT3-ITD+ AML patients. The OS was significantly prolonged in the quizartinib arm compared to the placebo arm. The median OS was of about 32 months with quizartinib *vs.* 15 months with placebo, while CR rates were 71.6% and CRi rates 64.9%[52,53].

Crenolanib, compared to other drugs of the same class, demonstrates some attractive characteristics to target FLT3 mutations in AML. Being a potent type I pan-FLT3 inhibitor, crenolanib is also active in FLT3 TKD mutations[54]. As a single agent, in R/R AML Crenolanib showed 39% of CRi and 11% of PR among the 18 patients treated[55], and in another study in 34 evaluable patients, 12% achieved CRi[56]. However, these responses to crenolanib were transient [Table 1].

MOLECULAR MECHANISMS OF RESISTANCE

The heterogeneity of FLT3-ITD mutations such as length, sequence, and duplication site is unique for every AML patient[57,58], and this molecular diversity increases the complex biology of FLT3-ITD. As previously reported, FLT3-ITD length mutations are found in the JM domain of the gene in 70% of mutated AML cases, while in the remaining 30%, the duplication region can involve the TKD1 with a different prognostic impact[30,59]. However, the molecular mechanisms responsible for the differential signaling based on FLT3-ITD sites are not completely understood and can also influence the clinical outcome of patients [Table 2]. The use of FLT3 inhibitors in clinical practice showed a good response rate in mutated AML, both as frontline and relapsed/refractory therapy, but about 30%-40% of patients underwent relapse due to the acquisition or clonal evolution of gene alterations that drive therapy resistance[17,60,61]. Therefore, resistance to FLT3 inhibitors can be categorized as primary and secondary resistance. During the innate resistance, administration of FLT3 inhibitors at AML diagnosis may be impeded in its efficacy by FLT3 ligand, fibroblast growth factor 2 (FGF2) and stromal cytochrome P450 3A4 (CYP3A4) mediated manner. It is well known that FLT3-mutated AML cells also express high levels of the wild-type FLT3 receptor, and therefore, FL ligand expression is consequently increased during the administration of FLT3 inhibitors. FL ligand binds to the FLT3 receptor and, in turn, restores FLT3 and downstream MAPK signaling, allowing the FLT3-ITD mutated clone to survive during the induction and consolidation therapy[62]. In this scenario,

Table 1. Clinical trials including targeted FLT3 Inhibitors in adult patients with AML

Type of treatment	Therapeutic combinations including FLT3 inhibitors	Trial Phase	ClinicalTrials.gov identifier	AML[1] setting
Chemotherapy + FLT3 inhibitors	"7 + 3" + gilteritinib *vs.* "7 + 3"	3	NCT02236013	*de novo* AML[1]
	"7 + 3" + quizartinib *vs.* "7 + 3"	3	NCT02668653	*de novo* AML[1] with mutated FLT3-ITD[2]
	"7 + 3" + crenolanib	2	NCT02283177	*de novo* AML with mutated FLT3
	CPX-351 + quizartinib	2	NCT04209725	R/R[3] AML[1] with mutated FLT3-ITD[2]
	"7 + 3" + crenolanib *vs.* "7 + 3" + midostaurin	3	NCT03258931	*de novo* AML[1] with mutated FLT3
	"7 + 3" + crenolanib *vs.* "7 + 3"	3	NCT03250338	R/R[3] AML[1] with mutated FLT3
	HD-Ara-c[5] + Mito[6] + quizartinib	2	NCT03989713	R/R[3] AML[1] with mutated FLT3-ITD[2]
	CdA[7] + ida[8] + Ara-c[9] + quizartinib	2	NCT04047641	*de novo* and R/R[3] AML[1]
HMAs + FLT3 inhibitors	Aza[10] or LD-Ara-c[11] + quizartinib	1/2	NCT01892371	Phase I cohort: R/R[3] AML[1] Phase II cohort: *de novo* and R/R[3] AML[1] with mutated FLT3
	Aza[10] + gilteritinib *vs.* aza[10]	3	NCT02752035	*de novo* AML[1] with mutated FLT3
Maintenance with FLT3 inhibitors	Crenolanib	2	NCT02400255	AML[1] with mutated FLT3 post-transplant
	Gilteritinib *vs.* placebo	3	NCT02997202	AML[1] with mutated FLT3 post-transplant
	Gilteritinib	2	NCT02927262	AML[1] with mutated FLT3 post CR1[4] achievement
New molecules + FLT3 inhibitors	Dec[12] + ven[13] + quizartinib	1/2	NCT03661307	*de novo* and R/R[3] AML[1] with mutated FLT3
	Ven[13] + gilteritinib	1	NCT03625505	R/R[3] AML
	Ven[13] + quizartinib	1/2	NCT03735875	R/R[3] AML with mutated FLT3
	Aza[10] + ven[13] + gilteritinib	1/2	NCT04140487	Phase I cohort: R/R[3] AML with mutated FLT3 Phase IIa cohort: *de novo* AML[1]
	Atezolizumab + gilteritinib	1/2	NCT03730012	R/R[3] AML[1] with mutated FLT3
	MTOR inhibitor (RAD001) + FLT3 inhibitor (PKC412)	1	NCT00819546	R/R[3] AML[1]
	Milademetan (MDM2inh.) + quizartinib	1	NCT03552029	*de novo* and R/R[3] AML with mutated FLT3
	"7+3" + GO[14] + midostaurin	1	NCT03900949	*de novo* AML[1] with mutated FLT3

AML: Acute myeloid leukemia; Ara-c: cytarabine; Aza: 5-azacitine; CdA: cladribine; CR1: first complete response; Dec: decitabine; GO: gemtuzumab ozogamicin; HD-Ara-c: high dose cytarabine; ITD: internal tandem duplication; Ida: idarubicine; LD-Ara-c: low dose cytarabine; Mito: mitoxantrone; R/R: relapsed/refractory; Ven: venetoclax.

FGF2 also plays an important role during TKIs resistance. Several studies have demonstrated that FGF2 is highly expressed in bone marrow stromal cells and its binding to FGFR1 receptor in FLT3-ITD mutated cells promotes resistance to FLT3 inhibitors through the activation of the downstream MAPK pathway[63]. Finally, loss of FLT3 inhibitors efficacy may arise due to insufficient drug concentrations in plasma, as a consequence of its rapid hepatic metabolism and in BM stromal cells, by cytochrome CYP3A4 enzymes[64]. Secondary resistance to FLT3-TKIs occurs through a variety of mechanisms, and in most cases, patients gain secondary resistance either by acquiring on-target or off-target abnormalities[65]. The majority of relapsed/refractory patients display on-target FLT3 alterations (26%), acquiring the D835, N676, F691, and Y862 mutations within the TK domain, the most common secondary events identified in AML patients treated with type II FLT3-TKIs[66]. Off-target mutations, which account for 16% of refractory patients, can occur *de novo* in primary FLT3-mutated clones or be gained by the evolution of other neoplastic clones. These cases are frequently characterized by gene mutations of epigenetic modifiers (DNMT3A, TET2, IDH2). Further genetic mutations leading to an off-target resistance after therapy with FLT3-TKI have been

Table 2. Proposed mechanisms of resistance to FLT3 Inhibitors in AML

Mechanism	Description	References
	Primary resistance	
Cytochrome P450 3A4-mediated degradation of FLT3-inhibitors	Plasma levels of FLT3 inhibitors can be decreased through their enhanced degradation by hepatocytes and BMSC in Cytochrome P450 3A4-mediated manner	[64]
Restoration of FL/FLT3 signaling through compensatory overexpression of wild-type (WT) FLT3 receptor and FL	FLT3-mutated AML increases the expression of FL binding to WT FLT3 receptor and restores the downstream FLT3-MAPK signaling in WT and FLT3-ITD co-mutated cells	PMID: 27331411 PMID: 21263155
Activation of common FLT3 and fibroblast growth factor receptor type 1 (FGFR1) downstream target pathways through the increased fibroblast growth factor 2 (FGF2)/FGFR1 signaling	FGF2 ligand highly expressed in BMSC binds to FGFR1 receptor in FLT3-ITD mutated cells and activates the downstream MAPK signaling shared by both receptor tyrosine kinases	[63]
Secondary resistance		
Newly acquired activating mutations of FLT3 TKD	Acquired mutations (D835, N676, F691 and Y862, A627 and others) within FLT3 TK domain confer resistance to different TKI and sustain activation of the downstream effectors	[66,71] PMID: 22858906 PMID: 33780043
Acquired activating mutations in RAS/MAPK pathway genes	Activating mutations in RAS/MAPK pathway genes upregulate pro-survival and pro-proliferative mechanisms in leukemic cells	[66,67,69,74]
Acquired mutations in epigenetic regulators and transcriptional regulators	Acquired mutations in epigenetic modifiers like DNMT3A, TET2, IDH2 or transcriptional regulators like WT1 and TP53 upregulate pro-survival mechanisms and favor leukemic cells survival	[60,67,76]
GM-CSF and IL-3 maintain cell survival without rescuing proliferation	Cytokine-mediated resistance through GM-CSF and IL-3 is dependent on JAK kinase, STAT5, and pro-viral integration site of Moloney murine leukemia virus (PIM) but not MAPK or mammalian target of rapamycin signaling	PMID: 30944098
Novel ATM/mTOR pathway regulating oxidative phosphorylation	Marrow-mediated activation of ATM, in turn, upregulates oxidative phosphorylation via mTOR signaling. mTOR is required for the bone marrow stroma-dependent maintenance of protein translation	PMID: 36259537

AML: Acute myeloid leukemia; BMSC: BM stromal cells; FLT3: FMS-like tyrosine kinase 3; TKD: tyrosine kinase domain.

recently reported in genes of the RAS/MAPK pathway (13%), WT1 (7%), and TP53 (7%)[66,67]. There is hope that recognizing and understanding the mechanisms that produce FLT3-TKIs resistance will help provide better strategies for the rational design of new agents and, finally, lead to more effective treatment [Table 2].

Acquired mutation on *FLT3* gene

Several studies have demonstrated that TKIs drugs may have an impact on the clonal evolution of FLT3-ITD mutated AML by downregulating certain clones[68,69]. These findings emphasize the importance of repeated mutation analysis for FLT3-ITD to discriminate between patients in whom TKI may induce long-lasting remission, and from those in whom relapse may originate from subclones, which may carry a FLT3-wild type at diagnosis. As previously discussed, secondary TKD mutations, mainly reported at residues D835/F691 in FLT3-ITD mutated patients treated with TKIs, confer therapy resistance and poor outcomes[70]. In particular, at AML onset, type II of FLT3-TKIs have an effect on FLT3-TKD mutations, but secondary alterations in the TK domain during the disease progression may confer treatment resistance interfering with the inhibitory activity on FLT3-ITD mutated clones. Acquired alterations at D835, F691 and Y842 residues have been reported in patients who developed resistance to sorafenib or quizartinib, both type II-TKIs[71]. Although the acquisition of mutations in the TK domain of FLT3 is rarely reported in patients treated with gilteritinib and crenolanib (a type I-TKIs), the gaining of F691L alteration, defined as gatekeeper mutation of FLT3 gene, was frequently observed. This mutation confers substantial resistance to both type I and type II-TKIs[65]. The latter mutations occur in residues that directly interact with the TKIs and become prevalent in AML clones almost exclusively after treatment with the inhibitors[72]. Although in FLT3-mutated AML, the addition of midostaurin to standard "7 + 3" treatment regimen has been widely

used in clinical practice, only 60% of patients achieved a CR, and almost half of these cases developed a relapse. Schmalbrock and colleagues[16] provided novel biological features into the clonal evolution and mechanisms of resistance of FLT3-ITD-mutated AML exposed to midostaurin. They demonstrated that during the disease progression, almost 46% became FLT3-ITD negative and acquired mutations in MAPK pathways, conferring an additional proliferative advantage. By contrast, in AML cases that relapse with FLT3-ITD persistence, they showed a clonal selection of driver mutations in 11% of cases. FLT3-ITD clones persist in the remaining patients during relapse disease, indicating a failure of midostaurin inhibition activity.

Intracellular pathways alterations

The phosphorylation of FLT3 receptor activates several downstream intracellular signaling pathways, such as RAS/MAPK, PI3K/Akt/mTOR, and JAK/STAT5, which are mainly implicated in the survival, proliferation, and differentiation of hematopoietic cells[73]. During the TKI treatment, clonal selection of cells characterized by activating gene mutations involved in RAS/MAPK pathway is often detected during progression in AML patients who received frontline midostaurin combination therapy[69] or gilteritinib as monotherapy for relapsed/refractory disease in 2nd line[67,74,75]. These results suggest that RAS mutations may drive the clonal evolution in relapsed/refractory AML that occurs independently from TKI type administration. Other mechanisms of off-target resistance during FLT3-TKI therapy have been recently described. Alotaibi and colleagues[67] analyzed the mutational status of patients who relapsed after different FLT3 inhibitors and demonstrated that the most common gene alterations involved alterations in IDHs, NRAS, WT1, and TP53 genes. In particular, they identified in responding patients a higher occurrence of IDH2 alterations at diagnosis as compared to non-responder cases. This data suggests an advantage of combining targeted therapies in AML patients who harbored concomitant FLT3 and IDH2 mutations[76]. Instead, mutations of NRAS and IDHs genes often occur in leukemia subclones; TET2 alterations are described in FLT3 mutated clones and mainly enriched in crenolanib poor-responders AML. In particular, Zhang *et al.* reported that the mutation type of TET2 gene correlates with a different prognosis in patients treated with crenolanib, suggesting that TET2 truncation mutations may contribute to TKI resistance as compared to missense mutations that did not show a correlation with an unfavorable response to crenolanib[60].

CLINICAL RELEVANCE OF TKI RESISTANCE

It has been demonstrated that FLT3 inhibitor therapy promotes drug-resistant clonal populations that harbor secondary, on-target FLT3-mutations and that are prone to resistance to numerous TKIs in patients with relapsed AML who possess *FLT3-ITD* mutations[77]. Prolonged therapy with FLT3-TKIs can exert clonal pressure for the selection of drug-resistant sub-clones with additional mutations that facilitate leukemic proliferation regardless of the *FLT3* kinase's activation state, as well as for sub-clones that are completely lacking FLT3 mutations; this is particularly relevant because relapsed AML is demonstrably a polyclonal disease[69].

Sorafenib and midostaurin, FLT3 TKIs of 1st-generation, have in proportion a low selectivity for *FLT3* mutations. Nevertheless, the rates of response are augmented with midostaurin and relapse rates show a significant reduction when either drug is administered in association with frontline chemotherapy, like 3 + 7 induction therapy in patients with newly diagnosed (ND-AML), FLT3-^{mut} AML[31]. *FLT3-ITDs* are patient-specific, and due to the unique positioning and variable extension of the duplicated genomic sequence, there is a wide range of variability. This translates into unique peptide motifs derived from duplicate aminoacidic sequences within the *FLT3* gene. Therefore, many studies have tried to answer the question if diversity of *FLT3-ITD* exerts any effect on the clinical outcome of AML patients[30,78-81]. In a study conducted

on 151 elderly AML patients who had received standard chemotherapy, Stirewalt *et al.* showed that the length of the ITD (40 *vs.* > 40 base pairs) had an effect on the patients' long-term survival[78]. According to research by Kayser and colleagues, patients with AML who have *FLT3-ITD* placed into the first tyrosine kinase domain (TKD1) of the *FLT3*-gene present a poorer outcome than those who have *FLT3-ITD* in the juxtamembrane domain (JMD)[30]. However, a retrospective analysis in 260 patients with AML *FLT3-ITD* positive, which divided *FLT3-ITD* into 3 groups based on its position, was unable to confirm this finding, and just showed a statistical trend towards a link between a more C-terminal location of *FLT3-ITD* and worse survival, but without impacting on EFS[79]. However, Fisher *et al.* reported data favorable to the assumption that *FLT3-ITDs* located closer to the C-terminus of the FLT3 gene correlate with an adverse prognosis[80]. They showed that the localization of the ITD affected the percentage of remission following AML first-line chemotherapy, independently of the allelic burden[80]. Rucker *et al.* assessed the prognostic and predictive role of *FLT3-ITD* insertion site (IS) considering 452 subjects treated in the RATIFY trial, identifying 265 IS in the tyrosine kinase domain-1 (TKD1) and 43 IS in the juxtamembrane domain (JMD) by NGS[81]. Four-year OS probabilities significantly differed between JMDsole, JMD/TKD1, and TKD1sole, respectively; specifically, multivariate Cox models for OS and cumulative occurrence of relapse after HSCT identified TKD1sole as a negative prognostic factor[81].

Gilteritinib and quizartinib, members of the second-generation FLT3 TKIs, show higher selectivity for FLT3 mutations. Indeed, when they are used as single agents in patients with FLT3-mutated R/R AML, both molecules exhibit clinical activity and have shown survival advantages over salvage approaches[17,61]. In comparison with quizartinib, gilteritinib showed activity for *FLT3-ITD* and *FLT3-TKD* mutations[82]. Nevertheless, secondary resistance to gilteritinib can arise via off-target mechanisms, such as the appearance of *NRAS* or similar mutations that activate MAPK signaling downstream of FLT3, as well as on-target FLT3 mutations at a gatekeeper FLT3 residue (F691L)[40,74]. As already discussed, gilteritinib has been approved as single-agent therapy for patients with FLT3-mutated R/R AML after positive results of ADMIRAL trial[61,79]. Patients randomized to receive 120 mg of gilteritinib showed a significantly longer median OS than those who received salvage chemotherapies and higher rates of CR[17]. Furthermore, Perl *et al.*[39] confronted post-hoc the clinical outcomes in patients with R/R FLT3-mutated AML enrolled in CHRYSALIS and ADMIRAL trials[17,39], with a view on those who were previously exposed to midostaurin or sorafenib against naive patients. Similar to those pre-treated, high rates of overall response emerged among patients treated with a FLT3 TKI before gilteritinib (CHRYSALIS, 42%; ADMIRAL, 52%) and patients without previous FLT3 TKI therapy (CHRYSALIS, 43%; ADMIRAL, 55%). Furthermore, in ADMIRAL study, a higher rate of response and a trend toward longer median OS was observed in the arm with gilteritinib *vs.* salvage chemotherapies in patients who had previously received a FLT3 TKI[83]. Therefore, these results encouraged the use of gilteritinib in FLT3-mutated R/R AML also after prior exposure to sorafenib or midostaurin.

POSSIBLE STRATEGIES TO OVERCOME RESISTANCE

New therapeutic combinations are now being tested in order to potentially reduce the development of resistances to FLT3 inhibitors or the possible occurrence of new point mutations within the FLT3 gene during the therapeutic approaches; this would allow an increased rate of response to treatment and a lower cumulative incidence of relapse in FLT3 mutated patients.

A phase 1 study (NCT02236013) assessed the safety, tolerability, and efficacy of gilteritinib plus 7 + 3 induction, followed by consolidation chemotherapy with high-dose ARA-C, and then a period of maintenance with gilteritinib in adults with ND-AML. The study included 80 patients; the rate of composite CR was 81.8% for all the four doses and 81.6% for patients who received 120 mg of gilteritinib (that represented the maximum tolerated dose). The median follow-up for OS was 35.8 months, with a mOS for

patients with FLT3-mutated disease that was not reached. Serious treatment-related adverse events (AE) took place in 10 patients; the most common nonhematologic AE of grade ≥ 3 were increased liver enzymes, pneumonia, and sepsis/bacteremia[84]. Recently, the MD Anderson group presented preliminary data from a phase II trial that evaluated the combination of gilteritinib with intensive chemotherapy (CLIA = cladribine, cytarabine and idarubicin) in ND-AML with *FLT3* mutation. Twenty-four patients were enrolled; 13 patients (54%) achieved a CR and underwent an allogeneic SCT[85]. Furthermore, treatment results for those patients with ND-AML, *FLT3*-mutated considered ineligible for a course of intensive chemotherapy are strongly disappointing. In a pooled analysis of the phase IB and phase III VIALE-A study (HMA + venetoclax), the median OS in FLT3 mutated patients was only 12.5 months, lower than what the 15 months achieved in FLT3 unmutated patients with this regimen[86]. Moreover, a phase III trial randomized (with a 2:1 ratio) untreated adults with FLT3 mutated AML unfit for intensive induction chemotherapy to gilteritinib (120 mg/day orally) and azacitidine (AZA + GIL) or azacitidine (AZA) alone. In the interim analysis, 123 patients were randomized (AZA + GIL, n = 74; AZA, n = 49). The median OS was not significantly different between the two arms (9.82 *vs.* 8.87 months, respectively; P = 0.753)[87]. As the median OS was the primary endpoint of the trial, the study was closed. Moreover, combination therapies to overcome resistance with FLT3 inhibitors and alternative resistance factors like apoptosis (BCL2) have been developed using venetoclax[88]. Preclinical data suggest that gilteritinib, inhibited the expression of BCL2A1 through the inactivation of STAT5 and alleviated TKI resistance of FLT3-mutated cell lines. Combining gilteritinib and venetoclax that suppresses BCL2A1 could improve the prognosis of AML with FLT3-ITD/D835 mutations[89].

Some patients harbor *IDH* and *FLT3* mutations at the time point of diagnosis or experience a second mutation while undergoing treatment. Particularly *FLT3* mutations are linked to low IDH inhibitors response rates, and treatment-emergent *FLT3* mutations also seem to impart therapeutic resistance to these drugs. As a result, there is increased interest in researching combined therapies that target multiple pathways; however, there is currently no information available regarding the clinical experience of combined IDH inhibitors and FLT3 inhibitors therapy. A retrospective analysis identified 12 patients who received concurrent IDH and FLT3 inhibitors therapy, 11 of whom had R/R AML. The composite remission rate was 33%, and the ORR (CR + CRi + MLFS) was 42%, while the treatment combination was well tolerated[76]. Therefore, a notable proportion of R/R AML patients benefitted from concurrent FLT3 and IDH inhibitors therapy and studies are ongoing to investigate this promising combination.

In FLT3-ITD AML preclinical models, FLT3-ITD inhibition in combination with venetoclax exhibits impressive anti-tumor effectiveness and offers a solid molecular basis for clinical trials. However, the use of selective BCL-2 family inhibitors revealed a new function for BCL-2, BCL-XL, and MCL-1 in FLT3-ITD positive cells' *in vivo* survival, underscoring the necessity of targeting all three proteins for the most effective anti-tumor effect[88]. An open-label, phase 1b trial (NCT03625505) evaluated the combination of venetoclax and gilteritinib in R/R AML. Among the 54 patients enrolled, 38 patients (74.5%) achieved a response, with a median OS and a median duration of response of 10.5 and 5.6 months, respectively. In a post hoc analysis of the 30 analyzable patients who had achieved a CR with at least one follow-up MRD assessment, 17 (56.7%) achieved molecular clearance defined as FLT3 allelic burden < 10^{-2}[90]. The MD Anderson group hypothesized that triplet therapy combining FLT3 inhibitors, venetoclax, and hypomethylating agents (HMAs) would further improve outcomes of FLT3 mutated patients. Therefore, they added FLT3 inhibitors to a regimen of 10-day decitabine with venetoclax in newly diagnosed (ND) and R/R FLT3 mutated patients. In ND patients, the composite complete remission (CRc) rate was 92%, with MRD negativity by flow cytometry in 56% and by next-generation sequencing (NGS) in 91% of responders. In R/R AML, the CRc rate was 62%, with MRD negativity rate by flow cytometry in 63% and by NGS in 100% of

responders[91]. Therefore, triplet therapy with FLT3 inhibitors, venetoclax, and decitabine seems safe and an excellent frontline option for older patients with ND FLT3 mutated AML, and effective for R/R AML. A transition to allogenic transplant and post-transplant maintenance with FLT3 inhibitors would offer further improvement in long-term outcomes.

Although midostaurin and gilteritinib have been given FDA approval to treat patients with FLT3-mutations, the effectiveness of these treatments is still constrained by the fact that half of patients die during the first five years from diagnosis. Our actual understandings of the limits of TKIs highlight the demand for alternative therapeutic approaches. Although FLT3 antigen density on AML blasts is substantially lower than - for example, CD20 on lymphoma cells - and is not thought to be sufficient for producing powerful antibody-mediated effector functions, the membrane receptor FLT3 has also been studied as a target for immunotherapy using monoclonal antibodies. AML blasts and normal hematopoietic stem cells (HSCs), to a lesser extent, are selectively bound by the mouse anti-human FLT3 monoclonal antibody (mAb) 4G8. In preclinical models, 4G8 conferred selective reactivity against AML blasts with high FLT3 antigen density after Fc tuning[92]. Recently, T-cells engineered to express a FLT3-specific chimeric antigen receptor (CAR) were produced and it was shown that they confer robust reactivity against both AML cell lines and primary AML blasts, that express either wild-type FLT3 or FLT3-ITD. Furthermore, it was observed that treatment with crenolanib produced an increased surface expression of FLT3, particularly on *FLT3-ITD* + AML cells. Therefore, it enhanced the recognition by FLT3-CAR T-cells in vitro and *in vivo*[93]. Sommer *et al.* reported the preclinical evaluation of another off-the-shelf CAR-T cell construct targeting *FLT3*[94]. This CAR construct with single-chain variable fragments (scFvs) was directed against multiple *FLT3* extracellular epitopes and was investigated for its capacity to direct T-cell selectivity and effector function to FLT3-mut AML cells. AML initial blasts are eliminated by allogeneic FLT3 CAR T cells made from a group of voluntary donors of T-cells; however, these cells are also effective against mouse and human hematopoietic stem and progenitor cells, raising concerns about myelotoxicity. Authors demonstrated that rituximab-mediated reduction of FLT3 CAR T cells following AML eradication permitted bone marrow regeneration without compromising leukemia remission by using a surrogate CAR with an affinity for murine FLT3[94].

Moreover, bispecific antibody cross-linking of FLT3 and CD3 demonstrated potent anti-leukemia activity against FLT3-mut cells. An anti-FLT3-CD3 immunoglobulin G (IgG)-based bispecific antibody (7,370) with a high affinity for FLT3 and a long half-life was developed to target FLT3-expressing AML blasts regardless of *FLT3* mutational status. In vitro and *in vivo* testing revealed that 7,370 exhibits picomolar potency against AML cell lines. Additionally, 7,370 was able to stimulate T cells from AML patients, refocusing their cytotoxic activity at low effector-to-target ratios on autologous blasts[95].

Finally, cyclin-dependent kinase 6 (CDK6) is a protein that works as a transcriptional factor, regulating FLT3 and the serine-threonine kinase PIM1, a different step in the process of leukemogenesis[96]. Uras *et al.* found that FLT3-mutant AML cells are very responsive to palbociclib, one of several available inhibitors of CDK4/6 kinase[97]. They showed that the cell cycle kinase CDK6 is required for the viability of FLT3-dependent blastic cells and that FLT3-ITD could induce leukemogenesis [Figure 2][97].

CONCLUSIONS

One of the most important modifications introduced with the current ELN2022 is that the *FLT3*-ITD allelic ratio is no more included in the ELN risk classification; as a consequence, AML with *FLT3*-ITD (without other adverse-risk genetic lesions) are now comprised into the group of the intermediate-risk and not the high-risk group, this independently from the allelic-ratio or the concurrent presence of *NPM1*-mut. The reasons for this modification are related to methodological difficulties in standardizing the assay adopted to

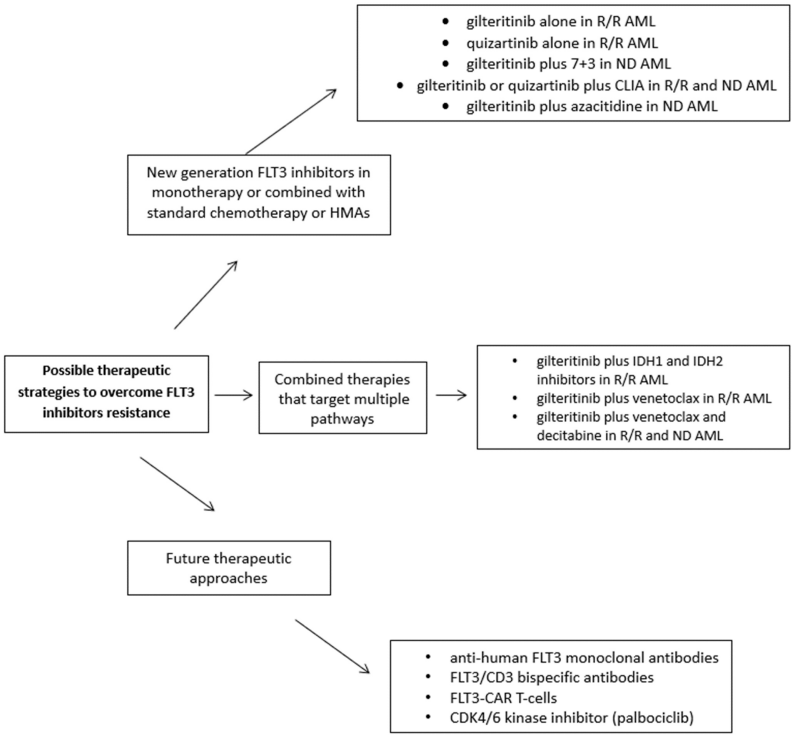

Figure 2. Possible strategies to overcome FLT3-inhibitor resistance.

measure the *FLT3*-ITD allelic ratio in different labs, and the improved effect of midostaurin-based chemotherapy on AML patients with *FLT3*-ITD without *NPM1*-mut[55,98]. Several mechanisms of resistance to available FLT3-inhibitors have been partly elucidated, but it is a process that acts underneath many biologic fields (e.g., the emergence of antibiotic-resistant strains in many bacteria), also with a clonal selection of resistant sub-clones of AML after protracted biological pressure from TKI-inhibitors. Nevertheless, in the ADMIRAL trial, patients who achieved a CR experienced a median duration of this response of 23.0 months, suggesting that the effect is protracted in a subset of patients. However, the cumulative effect of these drugs on patients with AML harboring FLT3-mutations has been demonstrated to be largely beneficial in clinical trials and current clinical practice. In Europe, we have only gilteritinib and midostaurin approved in clinical practice, so an exchange for a different TKI is not possible yet.

As a result, we reasoned, is resistance "Much Ado about nothing?" Probably, from the actual clinical perspective, mechanisms of resistance to FLT3-inhibitors are somehow overlooked. For patients obtaining a CR after therapy with gilteritinib, if they are transplant eligible, considering an early Allogeneic transplant seems appropriate because the duration of a CR is generally short-lived and gilteritinib may represent a bridge to transplant[99]. When gilteritinib is given after Allo-transplantation, it results in a significant improvement in overall survival. In brief, exposure to gilteritinib for a limited interval after allo-SCT enhances Graft-*vs.*-Leukemia effects against FLT3-ITD+ leukemia without exacerbating GvHD[100].

Poor results have been reported in patients with R/R FLT3-ITD AML, who were treated with standard salvage chemotherapy and had a median OS of 5.5 months and 1- and 5-year OS rates of 25% and 7%, respectively[101]. On the contrary, the use of single-agent FLT3 inhibitors has improved survival over chemotherapy in the relapsed setting[17]. The path of FLT3 inhibitors is marked by possible strategies to combine different drugs to overcome the emergence of resistance[91,93]. We hope that these ongoing studies will benefit many patients in the future.

DECLARATIONS

Authors' contributions

Designed the paper: Perrone S, Molica M
Prepared Figure 1, graphical abstract, and wrote legends: Zhdanovskaya N
Wrote the paper and critically revised it: Perrone S, Molica M, Ottone T

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Editorial

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Cancer stem cells in drug resistance: an introduction to the e-book covering the special issue on the "Cancer Stem Cells and Drug Resistance"

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A major problem in current cancer drug treatment is that a subset of cancer cells in many cases survives even highly efficient chemotherapy, and after a while, these remaining cells cause a recurrence of the disease. Moreover, the re-emerging cancer cells often show a multidrug-resistant phenotype, higher proliferation rate, higher invasiveness, and increased metastatic potential, as compared to original cancer. Cancer relapse and multidrug resistance are widely observed also in the most up-to-date, specifically targeted drug- or immunotherapies, causing an unsurmountable clinical inefficiency in cancer eradication.

A widely favored explanation for this relapse phenomenon is the presence of "stem cell-like" cancer cells, surviving therapy within the original tumor mass and then rapidly generating the recurring malignancy. This presumed tumor-initiating cancer stem cell (CSC) population is suggested to be responsible for increased apoptosis and chemoresistance, higher epithelial-mesenchymal transition (EMT) potential, and clinical cancer relapse and metastasis[1-6]. An alternative proposal suggests the presence of drug-tolerant persister cells (DTPC) with special genetic features among the cancer cells, and this drug-tolerant persister (DTP) cell population may be the source of cancer relapse[7,8].

The major conceptual difference between the two proposed sources of re-occurring tumor cells after drug treatment is discussed in detail in the key reviews by Clevers[9] and Borst[10]. In very short, the CSC concept envisions within the heterogeneous tumor (containing mostly variably differentiated cells) the presence of specific, drug-resistant, stem cell-like clonal cells. In contrast, the concept of the DTP cells suggests that some of the cells within the tumor mass can transiently and reversibly up-regulate mechanisms responsible for drug resistance, and the surviving cells variably regain their tumor-forming capacity[7,8].

Irrespective of the actual mechanisms, the clonal presence or transient formation of these practically immortal cells poses a major challenge for cancer therapy. Thus, the exploration of the basic biology and the clinical features of these CS or DTP cells may bring a new, successful era in cancer chemotherapy, including targeted therapies. Therefore, an elucidation of the key molecular mechanisms regulating CSC/DTP activities could help the development of novel biomarkers and therapeutic targets to predict or combat the most aggressive malignant tumors in patients[11-14].

The chapters in this special issue e-book aim to explore some of the important features, molecular background, phenotype, and clinical role of the CSCs or DTPCs. Among these, Safa[15] reviews the drug and apoptosis resistance in the tumor-initiating CSCs, how they display increased self-renewal potential, anticancer drugs and radiation resistance, and show an increased epithelial to mesenchymal transition (EMT) progression. Also, the potential specific treatments targeting CSCs are explored in this chapter. Hansen *et al.*[16] discuss the presence of leukemia stem cells in AML and focus on the cell surface markers and intracellular transcription factors that can distinguish these leukemia stem cells from normal hemopoietic stem cells. These biomarkers may significantly help to specifically eliminate the stem cell-like leukemic cells and thus improve leukemia treatment.

The chapter by Gupta *et al.*[17] specifically explores the potential role of CSCs in cancer resistance to immunotherapy. They describe surface markers that are differentially expressed on CSCs and potentially help them to escape from immune surveillance and immune cell-dependent killing. Moreover, they suggest that CSC-released cytokines and other metabolites may result in a decreased anticancer immune response in the tumor microenvironment. All these features may help to devise more effective anticancer immunotherapies.

Koltai[18] in his chapter raises an interesting issue and concept related to the role of pH in the development of the cancer multidrug resistance phenotype, and the potential use of already existing or repurposed drugs to modulate this resistance. In addition, a chapter by Kim *et al.*[19] deals with the potential use of herbal extracts to provide effective treatment in lung cancer, by reducing drug resistance and the growth of cancer stem cell-based tumors from an NSCLC model cell line.

It is hoped that this special issue will provide significant value in enhancing the understanding of the role of cells that have a major impact on clinical drug resistance in cancer.

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Authors' contributions

The author contributed solely to the article.

Availability of data and materials

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Cancer Drug Resistance

Original Article

Open Access

miR-16-5p enhances sensitivity to RG7388 through targeting *PPM1D* expression (WIP1) in Childhood Acute Lymphoblastic Leukemia

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Abstract

Aim: Given the encouraging results of the p53-Mdm2 inhibitor RG7388 in clinical trials and the vital function of miR-16-5p in suppressing cell proliferation, the aim of the present study was to investigate the combined impact of RG7388 and miR-16-5p overexpression on the childhood acute lymphoblastic leukemia (chALL).

Methods: miRTarBase and miRDB, along with KEGG and STRING databases, were used to predict miR-16-5p target genes and explore protein-protein interaction networks, respectively. B- and T-lymphoblastic cell lines, in addition to patient primary cells, were treated with RG7388. Ectopic overexpression of miR-16-5p in Nalm6 cell line was induced through cell electroporation and transfection of microRNA mimics was confirmed by qRT-PCR. Cell viability was evaluated using the MTT assay. Western blot analyses were performed to evaluate the effects of RG7388 and miR-16-5p upregulation on the protein levels of p53 and its downstream target genes in chALL cells. Paired sample t-test was employed for statistical analyses.

Results: MTT assay showed RG7388-induced cytotoxicity in wild-type p53 Nalm6 cell line and p53 functional patient primary cells. However, CCRF-CEM and p53 non-functional leukemic cells indicated drug resistance. Western blot analyses validated the bioinformatics results, confirming the downregulation of WIP1, p53

stabilization, as well as overexpression of $p21^{WAF1}$ and Mdm2 proteins in Nalm6 cells transfected with miR-16-5p. Moreover, enhanced sensitivity to RG7388 was observed in the transfected cells.

Conclusion: This is the first study indicating the mechanistic importance of miR-16-5p overexpression in chALL and its inhibitory role in leukemia treatment when combined with the p53-Mdm2 antagonist, RG7388. These findings might be useful for researchers and clinicians to pave the way for better management of chALL.

Keywords: Pediatric ALL, miR-16-5p, RG7388, *PPM1D*, p53

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is one of the most common blood cancers in children with a low incidence rate of *TP53* mutations at diagnosis, which highlights this type of malignancy as an attractive candidate for treatment with p53-Mdm2 antagonists. Although 85%-90% of patients respond to the treatment, there are subsets that are refractory to the therapy and relapse is prevalent amongst individuals who achieve complete remission[1,2]. For this reason, new strategies and treatments are necessary to overcome drug resistance.

Cancer treatment has been recently improving with the introduction of targeted therapies to achieve greater specificity and less cytotoxicity. Owing to the main function of p53 in the enhancement of cell cycle arrest, response to DNA repair and apoptosis, enormous efforts have been made to advance new cancer treatments based on p53-targeted therapy. p21 is a well-known determinant of cell cycle arrest, which increases following induction of p53 stabilization and its activity[3]. The incidence rate of *TP53* mutations at diagnosis is low in various types of blood cancer including ALL (5%-10%). Therefore, activation of the p53 pathway by non-genotoxic inhibitors of mouse double minute 2 homolog (Mdm2) is a promising strategy to improve cancer therapy in hematological malignancies, including ALL[4]. Recently, small molecule inhibitors of p53-Mdm2 interaction have been developed and entered into early-phase clinical trials for the treatment of diverse types of cancer comprising blood cancer[5-8]. Amongst those entered into clinical trials, RG7388 has passed phase II clinical trial[8]. Thus, the prediction of sensitivity to Mdm2 inhibitors and identification of mechanisms of resistance toward Mdm2 inhibitors would be helpful in stratifying patients who might benefit from these therapeutic agents.

p53 levels and its activity are regulated under a complex network of proteins and microRNAs (miRNAs) to maintain normal levels and proper function of p53. The oncogene Protein Phosphatase, Mg2+/Mn2+ Dependent 1D (*PPM1D*), known as wild-type p53-induced phosphatase 1 (*WIP1*), negatively regulates p53 through different mechanisms, including direct dephosphorylation of p53 and its subsequent inactivation, and dephosphorylation of other proteins involved in the regulation of p53 such as Mdm2 and p53 activating kinases[9]. Consistent with the inhibitory effect of Wip1 on the p53 pathway, *PPM1D* gene is amplified and overexpressed in different cancer types including leukemia, suggesting that Wip1 may be a potential therapeutic target for leukemia[10]. Recent studies have demonstrated that selective inhibition of Wip1 leads to increased DNA damage response and sensitivity to anti-cancer agents working through the p53 pathway[10-13].

microRNAs are a class of small RNAs ,with an average of 22 nucleotides in length, which interact with the partially complementary sequences in the 3′ untranslated region (3′ UTR) of the target mRNA and negatively regulate its expression[9,14]. Although not proven in childhood ALL, miR-16-5p appears to be a major negative regulator of Wip1 protein expression in some cancers, which affects p53-Wip1 feedback loop[13] and can regulate cell fate[12]. miR-16-5p interacts with the 3′-UTR binding site of the human *PPM1D*

gene and directly represses the Wip1 protein expression, a negative regulator of p53, thereby indirectly promoting p53 activity and its pathway[13]. Previous research showed that miR-16-5p feedback loop with p53 and Wip1 increases sensitivity to doxorubicin[13] and affects cell fate determination[12]. Moreover, aberrant expression of miR-16-5p has been reported in chronic lymphocytic leukemia (CLL)[15,16].

In the present study, it was hypothesized that cell lines and primary samples harboring functional p53 are more sensitive to RG7388 compared to those with dysfunctional p53, and ectopic overexpression of miR-16-5p in cells with functional p53 affects WIP1 mRNA levels, and consequently respond to the p53-Mdm2 antagonist RG7388 in a p53-dependent manner.

METHODS

miR-16-5p-target interaction databases and visualization of protein-protein interaction network

miRTarBase and miRDB validated microRNA-target interactions databases were employed to clarify the interaction between miR-16-5p and PPM1D expression[17,18]. The Kyoto Encyclopedia of Genes and Genomes database (KEGG) (http://www.genome.jp/kegg/) was used as a pathway database to find the p53 pathway map (map04115)[19]. To visualize protein-protein interaction network between the Wip1 and other proteins with strong confidence (0.700 interaction score), the STRING database (https://string-db.org/) was used[20].

Chemicals and reagents

The small-molecule Mdm2 inhibitor RG7388 (Idasanutlin) was purchased from SelleckChem (Cambridge, UK). RG7388 was dissolved in dimethyl sulfoxide (DMSO) to provide a 10 mM stock solution and stored in small aliquots at -20°C. miR-CURY LNA™ Universal RT microRNA PCR kit and miR-CURY LNA™ miRNA Mimics (HAS-MIR- 16 -5p & NEGATIVE CONTROL 5 MIRCURY LNA) were purchased from QIAGEN (Hilden, Germany).

Cell lines

The leukemic cell lines Nalm6 and CCRF-CEM were sourced from the Pasteur Institute (Iran) authenticated cell bank. The cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% (v/v) FBS (Gibco, USA) and 1% (v/v) penicillin/streptomycin and grown at 37 °C, 5% CO2 in a humidified atmosphere. The *TP53* status of Nalm6 cell line is wild-type (NALM6 ATCC CRL-3273™) and the CCRF-CEM cell line (CCRF-CEM ATCC CCL-119™) harbors heterozygous *TP53* mutations (c.524G>A; p.R175H, and c.743G>A; p.R248Q).

Patients, sampling and cell isolation

Peripheral blood or bone marrow samples (n = 10, 5 females and 5 males) from childhood ALL patients were collected. ALL in these patients was clinically diagnosed and pathologically confirmed by a clinical team through phenotypic, immunologic and cytogenetic techniques in the pediatric department of Sayed-ol-Shohada Hospital (Isfahan, Iran). Informed written consent was obtained in accordance with the Declaration of Helsinki, and with approval from the Ethics Committee of the University of Isfahan (ethics agreement number IR.UI.REC.1397.145). Informed written consent was obtained from the children's parents prior to participation in the study.

2-5 mL of heparinized bone marrow sample or peripheral blood were collected from patients and sent to the Cellular and Molecular Biology Laboratory of the University of Isfahan on ice. Mononuclear cells were extracted and isolated using density gradient Lymphoprep (Axis-Shailed Diagnostics Ltd, Oslo, Norway) according to the manufacturer's protocol.

Ex vivo cytotoxicity assay

Cytotoxicity of RG7388 was assessed using MTT cell proliferation kit (Alban, Austria). Growth curves were constructed for Nalm6 and CCRF-CEM cell lines (GraphPad Prism statistical analysis software version 8.) to measure cell doubling time and the optimum seeding density for a subsequent growth inhibition assay. Nalm6 (5×10^5/mL) and CCRF-CEM (4.5×10^4/mL) in 100 µL of medium per well of a 96-well plate were treated with a range of concentrations of RG7388 for 96 and 72 h, respectively (according to their cell doubling time). Then, 10 µL of MTT solution was added into the wells. 100 µL DMSO was added after 3 h to dissolve formazan crystals, and absorbance was measured using a stat fax 2000 microplate reader (Awareness Technology, Inc) at 492 nm wavelength. The LC_{50} values, the required concentration of each compound expected to kill 50% of the population, were determined using the statistical software mentioned above.

For patients` samples, primary cells (2×10^6/mL) in 100 µL of medium per well of a 96-well plate were exposed to 0.5% DMSO or 0.5 µM RG7388 ($2 \times LC_{50}$ concentration for Nalm6) for 72 h. This concentration was used since sensitive cells respond to it, and the sensitivity is not due to off-target effects. The proportion of the viable cells was measured by comparison between the absorbance of cells exposed to DMSO, as a control, or RG7388, and calculated using the following formula: (%) = [100 × (sample absorbance)/ (control absorbance)].

Functional assessment of the p53 pathway

To determine the functional status of p53 in ALL patients' samples (those with enough amount of protein lysates), the modulation of p53 and its transcriptional target gene protein products including Mdm2 and $p21^{WAF1}$ were evaluated following short-term exposure to Mdm2-p53 antagonist RG7388[21,22].

Western blotting

Nalm6 and CCRF-CEM (2.5×10^5/mL) were seeded in 2 mL culture media per well of a 6-well plate and treated with 0.5% DMSO and a range of concentrations of RG7388. Primary cells (0.5×10^6/mL) were also seeded in 2 mL culture media per well of a 6-well plate and exposed to 0.5% DMSO or 0.5 µM RG7388 ($2 \times LC_{50}$ concentration for Nalm6). Cells were harvested and lysed at 6 h. Lysis buffer (12.5 mL Tris HCL, 2 g SDS, 10 mL Glycerol, 67.5 mL Distilled Water) was applied to harvest the whole-cell lysates, followed by sonication. Bradford solutions (100 MG Coomassie Blue 250 G, 50 mL ethanol 96%, 100 mL ortho-phosphoric acid 85% and bringing volume to 1000 mL by adding distilled H_2O) were used to estimate the concentration of protein in the cell lysates utilizing NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

Hand-poured gradient gels were prepared using Bio-Rad mini gel casting apparatus, and two different acrylamide solutions were applied to separate proteins[23]. The separated proteins were transferred by perpendicular electrophoresis to a nitrocellulose Hybond™ C membrane (Amersham, Buckinghamshire, UK). Monoclonal mouse anti-human primary antibodies Actin 1:250 (#: C4: sc-47778, Santacruz Biotechnology, INC.), Mdm2 1:300 (#: OP46-100UG, Merck Millipore), p21 1:100 (#: OP64, Calbiochem), p53 1:250 (#: 2B2.68: sc-71817, Santacruz Biotechnology, INC.) and Wip1 1:200 (#: F-10: sc-376257, Santacruz Biotechnology, INC.) were used. Secondary goat anti-mouse HRP-conjugated antibodies (#: P0447/P0448, Dako) were applied at 1:1000. 5% milk/1XTBS-Tween (w/v) was used in order to dilute all antibodies. Enhanced chemiluminescence (GE Life Sciences, UK) and X-ray film (Fujifilm, India) were employed to visualize the proteins. Image J software (National Institute of Health, USA) was used to quantify and analyze the intensity of visualized bands, and the results were normalized to DMSO control.

Cell transfection

Harvested Nalm6 cells in the exponential growth phase were resuspended in RPMI-1640 (Gibco, USA) supplemented with 0.5% (v/v) FBS (Gibco, USA). 200 µL cell suspension containing 2.5 × 10^6 of cells were transferred into sterile electroporation cuvettes (0.2 cm gap, Bio-Rad, USA) separately (miR-16-5p mimic and negative control). miR-16-5p mimic and negative control (200 nm) (miR-CURY LNA™ miRNA Mimics, Qiagen) were separately added to the cuvettes in the hood just before electroporation, and the cuvettes gently swirled. Then, the cuvette was placed in the holder in the electroporation system (Eppendorf Multiporator®, Germany) at room temperature. Electroporation was performed in accordance with Multiporator® Transfection Protocol with minor changes [Voltage: 250 V, Time constant (τ): 40 µs, No. of pulses (n): 1] in order to specifically optimize protocol based on the cell type and genetic modifications. After the pulse, the cell suspension was allowed to stand in the cuvette for 5 to 10 minutes at room temperature. Finally, the cell suspension was transferred from the cuvette to 2 mL normal medium in a well of a 6-well plate and incubated for 48 h. In order to determine the impact of overexpression of miR-16-5p on sensitivity to RG7388, 48 h transfected cells were exposed to RG7388 (0.5 µM) for 6 h. Transfected cells were harvested to evaluate cell viability and silencing assessment.

RNA extraction and cDNA synthesis

Total RNA including preserved miRNAs was extracted from Nalm6 cells and primary samples using TRizol reagents (Invitrogen, California, CA) as per the manufacturer's recommendations. The quality of the RNA and its concentration was assessed with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA) by the ratio of 260nm:280nm. The complementary DNA (cDNA) for miR-16-5p was synthesized on 200 ng of total RNA using miR-CURY LNA™ microRNA PCR kit (QIAGEN, Germany) and the thermal cycler (GeneAmp PCR Systems, AB Applied Biosystems) according to the manufacturer's guidelines.

Quantitative RT-PCR (qRT-PCR)

Ectopic expression of miR-16-5p was confirmed by real-time PCR assay. qRT-PCR for miR-16-5p converted to cDNA was carried out using the miRCURY LNA SYBR Green PCR kit (Qiagen, Germany), with 50 ng/µL of the cDNA samples per 10 µL final reaction volume, on a Chromo4TM system (BioRad, Foster City, California) as described by the manufacturer. Primers for miR-16-5p quantitative RT-PCR were obtained from Qiagen, and RNU6 small nuclear RNA ((Exiqon, Denmark) was employed as endogenous control for data normalization. ΔΔCt Method was applied to perform data analysis.

Statistical Analysis

All the presented statistical tests were performed, applying GraphPad Prism version 8.4.3 software. The statistical paired t-test was employed to compare the mean of 3 paired biological repeats, and significant differences are defined as $P < 0.05$.

RESULTS

miR-16-5p is proposed to target WIP1protein affecting p53 pathway

The miRTarBase experimentally validated microRNA-target interactions database confirmed *PPM1D* as a target for miR-16-5p with strong evidence (Reporter assay, qRT-PCR and Western blot). In terms of miRDB target prediction database, miR-16-5p was at the top 20 miRNAs targeting *PPM1D* expression.

KEGG p53 pathway has shown Wip1 protein as one of the main negative regulators of p53 activity. In addition, Wip1 activates the main negative regulator of p53, Mdm2, and inactivates the kinases which phosphorylate and enhance p53 activity including CHK2 and ATM [Figure 1A]. Furthermore, STRING database confirmed the functional association between Wip1 and p53 and other proteins involved in p53

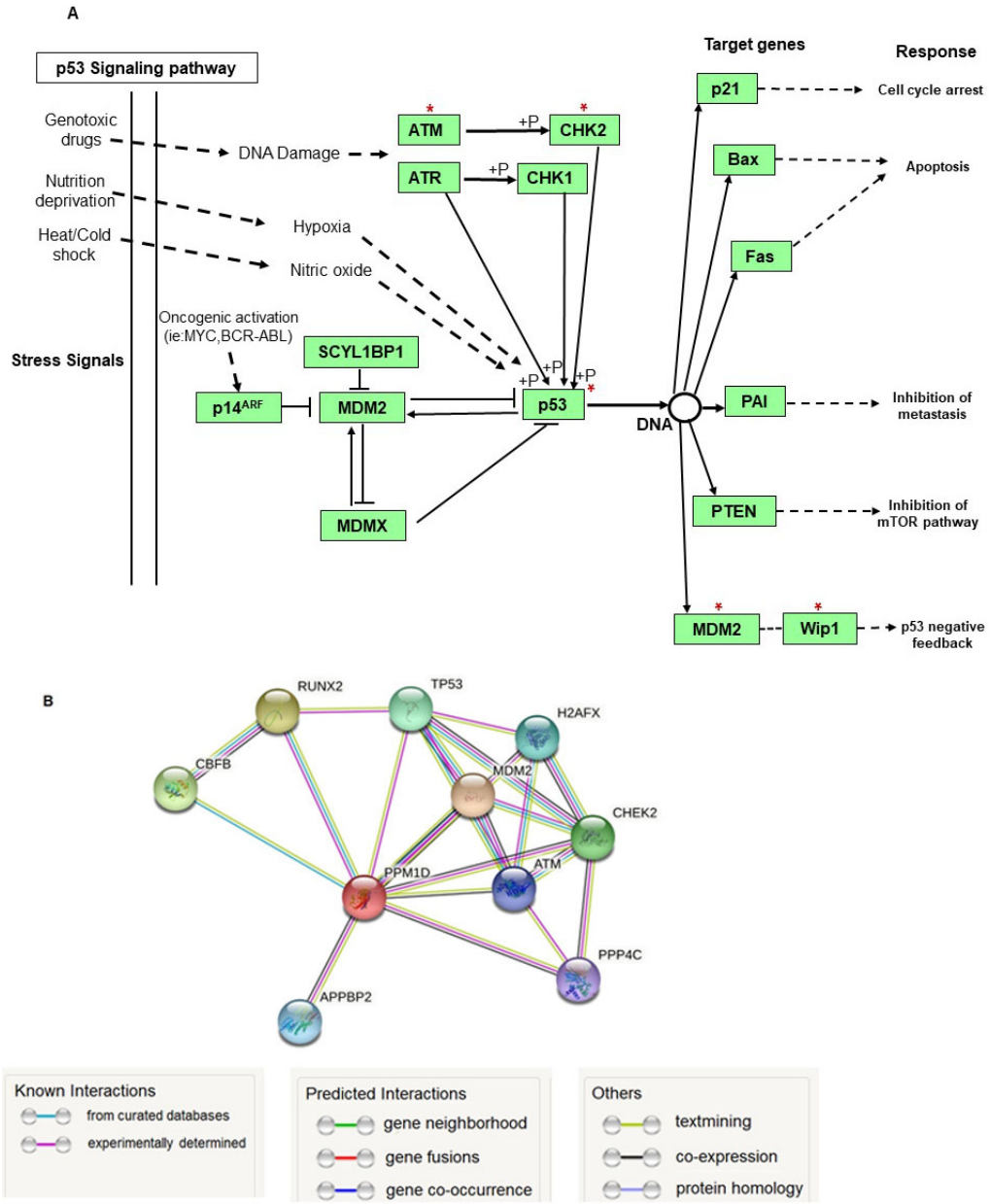

Figure 1. The importance of WIP1 in the p53 signaling pathway and its Protein–protein interaction network. (A) The p53 pathway map (map04115) was provided using the KEGG database. The red stars above each gene represent the genes whose activity is affected by WIP1 via dephosphorylation; (B) WIP1 Protein-protein interaction network was visualized by STRING with high confidence (0.7). The edges represent protein-protein associations which are meant to be specific and meaningful. This does not necessarily mean they are physically binding to each other. MDM2: Mouse Double Minute 2 Homolog; WIP1: Wild-type p53-Induced Phosphatase 1.

activity with a high confidence (0.7 score interaction) [Figure 1B].

The cytotoxic effect of RG7388 on ALL cell lines and primary cultures

The subsequent experiments were carried out to evaluate the sensitivity of two established ALL cell lines and ALL primary cells to RG7388, and the influence of ectopic overexpression of miR-16-5p on this sensitivity. The cytotoxicity of RG7388 and its p53-dependent effect was investigated using the MTT assay on Nalm6 wild-type *TP53* and CCRF-CEM mutant *TP53* cell lines. The LC_{50} values (Lethal Concentration

50%) indicated that wild-type *TP53* Nalm6 cell line was significantly more sensitive to RG7388 (0.27 ± 0.05 (SEM) μM) compared to CCRF-CEM mutant *TP53* cell line (> 2 μM) [Figure 2A]. The cytotoxic effect of RG7388 was also investigated on the cell viability of primary cultures generated from materials donated by ALL patients [Table 1]. 10 ALL samples were incubated with DMSO (0.5%) as control and RG7388 (0.5 μM), and they were examined for viability after 72 h using the MTT assay. RG7388 induced a cytotoxic effect on ALL cells, and 60% of those (6 out of 10) were sensitive and 40% (4 out of 10) were resistant to RG7388 with the delivered dose [Figure 2B]. RG7388 led to a significant reduction in the viability of sensitive ALL primary cells compared to their untreated counterpart ($P < 0.05$ or $P < 0.001$), with ALL 278 sample as the most sensitive sample (% viability = 0.66% ± 0.04, $P = 0.0005$). Overall, the median % survival for primary cultures was 53% [Figure 2C]. Notably, 3 out of 4 RG7388 resistant samples (75%) relapsed [Table 1].

Functional activation of the p53 pathway in ALL cell lines and primary cultures in response to RG7388

Functional assessment of the p53 pathway was evaluated by measuring p53 induction and its stabilization following six hours' exposure to RG7388, and consequent overexpression of its downstream targets including Mdm2 and $p21^{WAF1}$ proteins by Western blot. The p53-dependent response to RG7388 showed that RG7388 elevated p53 stabilization and overexpression of $p21^{WAF1}$ and Mdm2 protein levels 6 h after the commencement of treatment in a concentration-dependent manner, and confirmed functional activation of wild-type *TP53* Nalm6 cell line by release from Mdm2. However, as anticipated, it had no impact on the expression of p53-dependent genes in the *TP53*-mutant CCRF-CEM cell line with the delivered dose range of RG7388 [Figure 3A and Supplementary Figure 1].

For the primary cultures with sufficient amount of protein lysates for western blot, there was consistency between MTT assay results and p53 function. RG7388 induced functional stabilization of p53 and expression of its downstream target genes, $p21^{WAF1}$ and MDM2, in ALL samples that showed a significant decrease in their viability rate following treatment with RG7388. Conversely, there was no stabilization of p53 and induction of its downstream targets in primary cultures that were resistant to RG7388 [Figure 3B and Supplementary Figure 2].

RG7388 induces Wip1 expression in a p53-dependent manner

The basal protein levels of Wip1 and its expression levels following treatment with RG7388 at the 1 × and 2 × LC_{50} value for Nalm6 were determined in both Nalm6 and CCRF-CEM cell lines [Figure 3C and Supplementary Figure 3]. RG7388 increased stabilization of p53 protein, with subsequent increased expression of Wip1 protein in Nalm6 cells in a concentration-dependent manner. However, RG7388 failed to stabilize p53 and induce Wip1 in CCRF-CEM cells, indicating p53-dependent expression of Wip1 protein. Notably, both full-length (FL-WIP1) and its previously described shorter isoform (S-WIP1) of Wip1 protein were expressed by CCRF-CEM cell line.

miR-16-5p negatively regulates WIP1 expression and sensitizes Nalm6 cells to RG7388

The p53-Wip1 autoregulatory feedback loop regulates both expression levels of *PPM1D* gene and p53 activity. Previous studies showed that post-transcriptional regulation influences the expression of Wip1 protein and confirmed that miR-16-5p inhibits WIP1 expression through targeting 3'UTR of WIP1 [Figure 4A]. Thus, it is expected that miR-16-5p affects the response to RG7388 through regulating WIP1 in a p53-dependent manner.

To investigate the hypothesis, firstly, Nalm6 cells were transfected with miR-16-5p mimic or scrambled miRNA oligonucleotides as a negative control. Real-time PCR results confirmed high expression of miR-16-5p in Nalm6 cells resulting from the transfection of miR-16-5p mimic after 48 h. A significant

Table 1. Clinicopathological data for 10 samples of pediatric ALL

Patient's number/Variable*	Sex Age (years)	Peripheral blood/Bone marrow	*De novo*/ Relapsed	Pre-B cell/ T cell	Cytogenetics**	Response to RG7388
ALL 269	Boy (2)	Peripheral blood	*De novo*	Pre-B cell	No	Sensitive
ALL 270	Girl (2)	Bone marrow	*De novo*	Pre-B cell	No	Sensitive
ALL 271	Boy (3)	Bone marrow	*De novo*	Pre-B cell	No	Resistant
ALL 272	Girl (5)	Peripheral blood	Relapsed	Pre-B cell	No	Resistant
ALL 273	Boy (9)	Bone marrow	Relapsed	T cell	No	Resistant
ALL 274	Boy (6)	Bone marrow	*De novo*	Pre-B cell	T (12, 21)/ETV6-RUNX1	Sensitive
ALL 275	Girl (3)	Peripheral blood	*De novo*	Pre-B cell	T (1, 19)/TCF3-PBX1	Sensitive
ALL 276	Girl (5)	Peripheral blood	*De novo*	Pre-B cell	No	Sensitive
ALL 277	Girl (5)	Peripheral blood	Relapsed	T cell	No	Resistant
ALL 278	Boy (12)	Peripheral blood	*De novo*	Pre-B cell	No	Sensitive

*The blast proportion for all patients' samples was over 70%. **The most common cytogenetics (T (4; 11)/KMT2A-AFF1, T (9; 22)/BCR-ABL1, T (1; 19)/ TCF3-PBX1, T (12; 21)/ ETV6-RUNX1) were only analyzed.

increase was observed in the ectopic expression of miR-16-5p (by ~ 2.5-fold) compared with DMSO control, mock control, and negative control ($P < 0.05$) [Figure 4B].

We evaluated the protein levels of Wip1, p53, $p21^{WAF1}$ and Mdm2 in Nalm6 treated with RG7388 in the presence of miR-16-5p mimic and negative control in order to investigate the impact of altered levels of miR-16-5p on the expression of the proteins. The high expression of miR-16-5p in Nalm6 led to a significant increase in p53 protein levels (by ~ 3.0 fold) [Figure 4C, 4D], $p21^{WAF1}$ protein levels (by ~ 2.5 fold) [Figure 4C, 4E] and Mdm2 [Figure 4C] compared to Nalm6 transfected with miRNA oligonucleotides as a negative control and DMSO control ($P < 0.05$) irrespective of treatment with RG7388. Interestingly, ectopic overexpression of miR-16-5p significantly suppressed induction of WIP1 in Nalm6 treated with RG7388, while scrambled negative control had no effect on the expression levels of WIP1 after RG7388 treatment. Furthermore, overexpression of miR-16-5p caused a significant upregulation of p53 (by ~ 15.5-fold, $P < 0.01$) [Figure 4C, 4D] and its target genes, $p21^{WAF1}$ (by ~ 2-fold, $P < 0.05$) [Figure 4C, 4E] and Mdm2, in Nalm6 treated with RG7388 compared to negative control treated with RG7388. These results clearly demonstrated that miR-16-5p negatively regulates Wip1 protein levels, which consequently affects the p53 pathway and response to RG7388.

DISCUSSION

Acute lymphoblastic leukemia (ALL) is the most prevalent type of pediatric blood cancer in which *TP53* mutations are infrequent, less than 5%, at diagnosis but rise to about 10% in relapsed ALL[24]. Although complete remission (CR) is achieved for many patients at the end of the induction phase of treatment, relapse and drug resistance are major challenges in treating cancer[25]. Recently, p53-Mdm2 binding antagonists have been advanced to restore wild-type p53 function with subsequent induction of cell cycle arrest and apoptosis. These targeted therapeutic agents have shown *in vitro* promising results alone and in

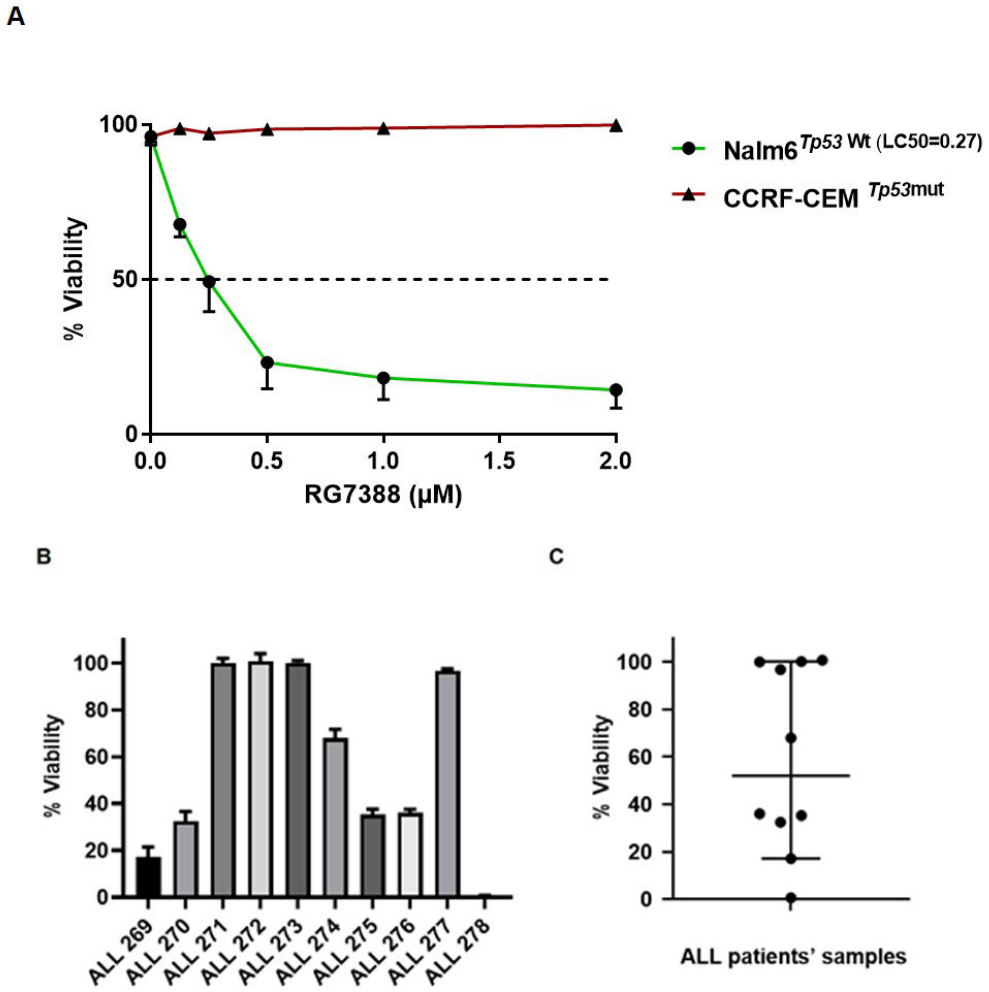

Figure 2. The sensitivity to MDM2 antagonists RG7388 in ALL cell lines and childhood ALL primary cells. (A) Wild-type *TP53* Nalm6 cell line is significantly more sensitive to growth inhibition by RG7388 treatment compared to mutant *TP53* CCRF-CEM cell line; (B) 10 pediatric ALL samples exposed to RG7388 (0.5 μM) for 72 h. RG7388 markedly decreased cell viability in most samples while assessed by MTT assay; (C) dot-plot of % viability for 10 pediatric ALL samples exposed to RG7388 (0.5 μM) for 72 h. Data shown are the average of three independent experiments and error bars represent SEM. ALL: Acute lymphoblastic leukemia.

combined treatments[26-31], and encouraging clinical trial outcomes in diverse types of cancer including blood malignancies[8,32-35]. miR-16-5p was previously reported as a post-transcriptional regulator of Wip1 in some types of cancer[13]. miRTarBase and miRDB microRNA-target interactions databases, KEGG pathway and STRING databases used in this study indicated the critical role of miR-16-5p in p53 activity through targeting PPM1D expression in ALL. Interestingly, the STRING database clearly showed strong interactions between Wip1 and p53 or its regulators comprising ATM, Mdm2 and CHK2 proteins[20].

The present study evaluated, for the first time, the impact of the Mdm2-p53 binding antagonist RG7388 in ALL cell lines, and primary cultures generated from materials donated by ALL patients. Moreover, this is the first study elucidating the positive effect of ectopic miR-16-5p on increasing sensitivity to RG7388 in *TP53* wild-type leukemic cells.

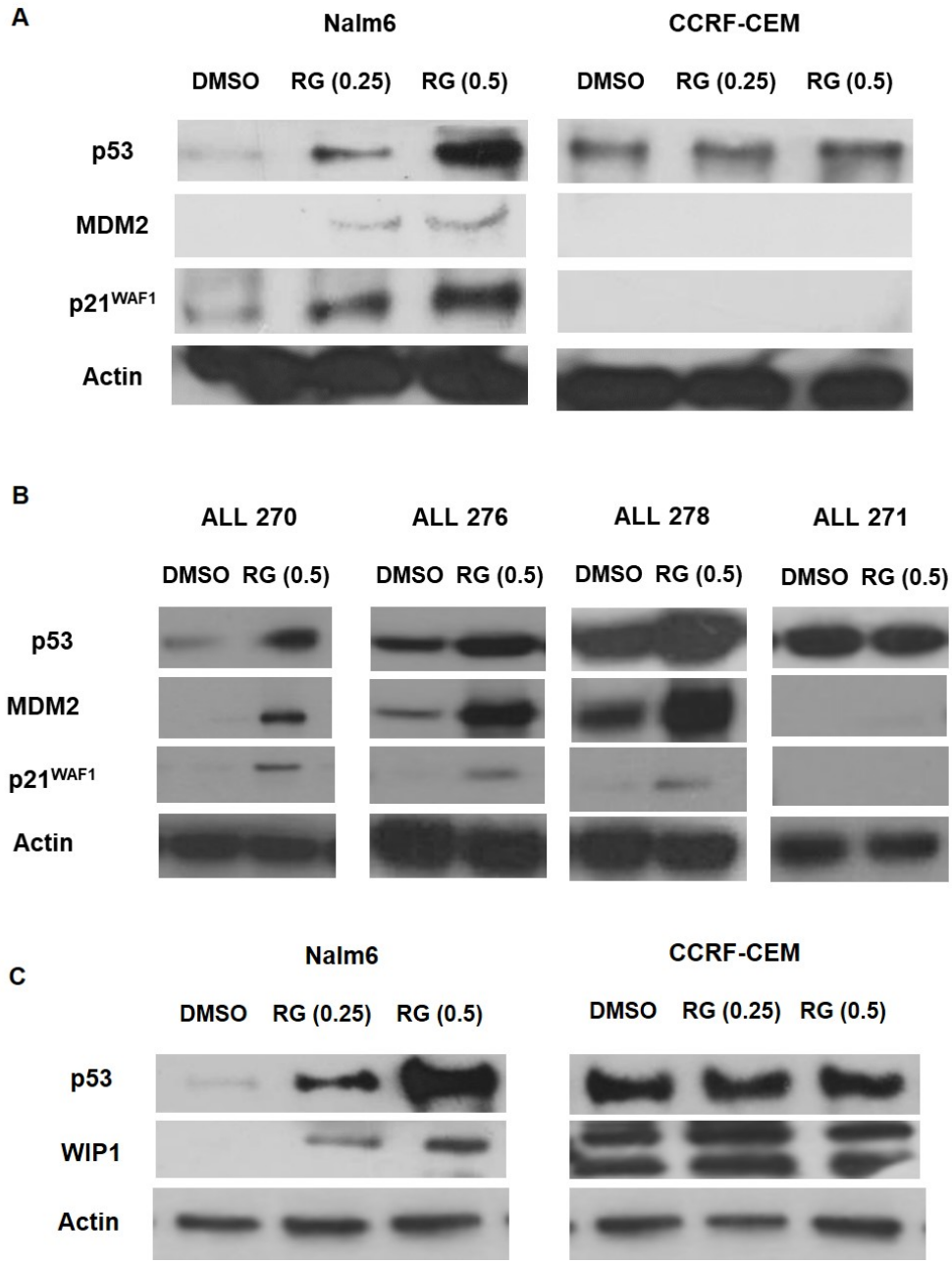

Figure 3. p53 functional stabilization in pediatric ALL cells in response to RG7388. Western blot analysis for (A) established Nalm6 and CCREF-CEM cell lines and (B) representative of patient samples with functional p53 and dysfunctional p53. RG7388 showed stabilization of p53 and upregulation of p53 transcriptional target gene protein levels, MDM2 and $p21^{WAF1}$, 6 h after the commencement of treatment in wild-type *TP53* Nalm6 and p53 functional ALL cells with the indicated doses (μM). However, it had no effect on downstream transcriptional targets of p53 in mutant *TP53* CCRF-CEM and dysfunctional p53 patient samples with the delivered dose of RG7388 (μM); (C) western blot analysis indicated stabilization of p53 and induction of WIP1 expression following 6 h treatment with indicated doses of RG7388 (μM) in wild-type *TP53* Nalm6 in a concentration manner. Conversely, there was no effect on the p53 stabilization and WIP1 expression in mutant *TP53* CCRF-CEM with the delivered dose range of RG7388 (μM). RG, RG7388. ALL: Acute lymphoblastic leukemia; MDM2: mouse double minute 2 Homolog; WIP1: wild-type p53-induced phosphatase 1.

Among the individual cell lines studied, wild-type *TP53* Nalm6 cell line was significantly more sensitive to RG7388 compared to mutant *TP53* CCRF-CEM cell line, which is in line with its mechanism of action[35]. These results partially confirm previous limited previous studies that indicated a significant decrease in the

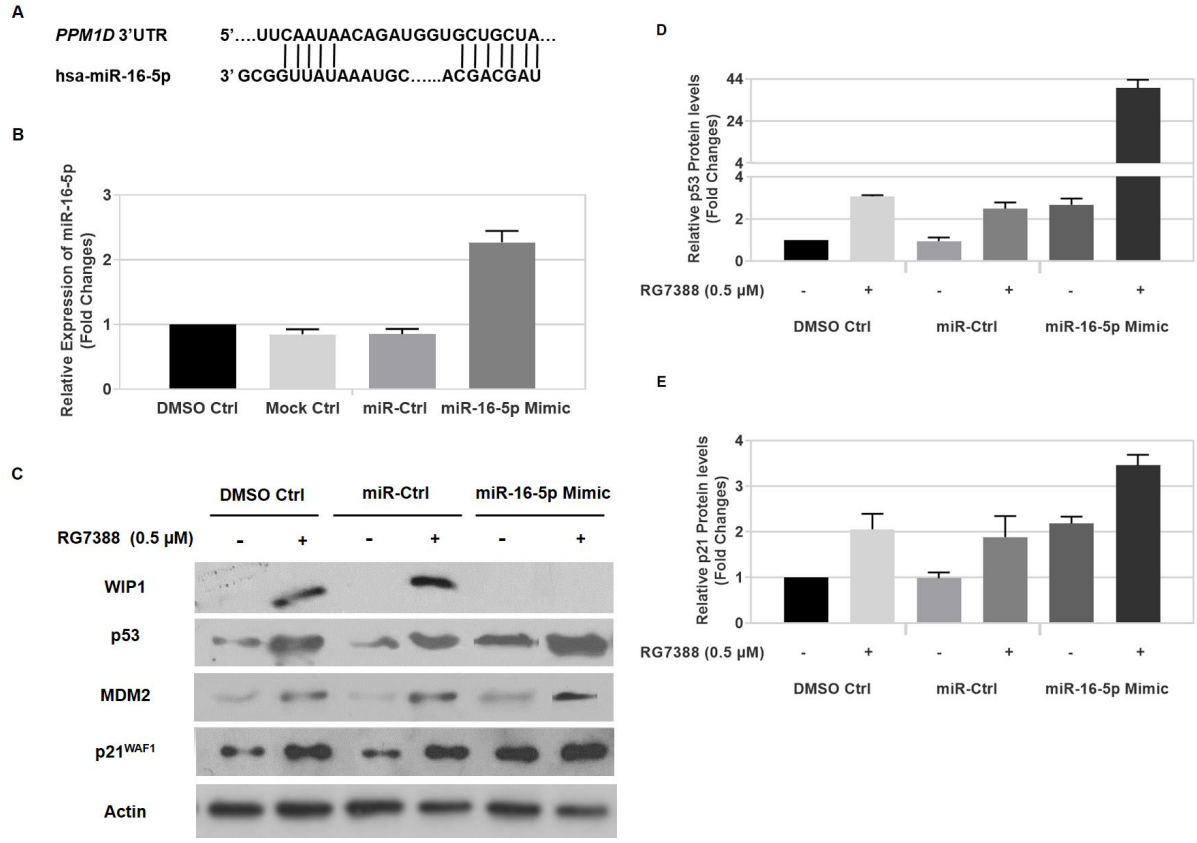

Figure 4. miR-16-5p suppresses WIP1 expression, affects p53 stabilization and its activity, and enhances sensitivity to RG7388. (A) miR-16-5p sequences and its putative binding sites in the 3'-UTR of PPM1D; (B) Nalm6 cells were transfected with negative control miRNA and miR-16-5p mimic (200nM). Total RNA was extracted from DMSO (0.5%) treated Nalm6, mock control, and those transfected with negative control miRNA or miR-16-5p mimic at 48 h after transfection. miR-16-5p levels were measured by quantitative RT-PCR shown significant ectopic overexpression of miR-16-5p in transfected cells with miR-16-5p compared to DMSO control, mock control and negative control miRNA; (C) ectopic overexpression of miR-16-5p suppresses WIP1 expression, enhances p53 stabilization and upregulates expression of p53 target genes, $p21^{WAF1}$ and MDM2 in wild-type *TP53* Nalm6 treated with RG7388. Nalm6 cells were transfected with miR-16-5p (200nM) or scrambled miRNA. Cells were treated with RG7388 (0.5 μM) at 48 h after transfection and their protein levels analyzed at 6 h after RG7388 treatment. The intensity of p53 blots ($P < 0.05$ or $P < 0.1$) (D) and $p21^{WAF1}$ blots ($P < 0.05$) (E) was quantified by Image J software and normalized with the DMSO control. Data shown are the average of three independent experiments and error bars represent SEM. Ctrl, Control. *$P < 0.05$, **$P < 0.01$

cell viability of wild-type *TP53* ALL cell lines following treatment with Mdm2 inhibitor Nutlin-3a[36,37].

Within a panel of primary cultures, RG7388 significantly decreased the viability of most ALL cells (6 out of 7, 86%) isolated from *de novo* patients. Conversely, primary cultures derived from relapsed patients were resistant to the cytotoxicity effect of RG7388 at the delivered dose. Given the fact that response to RG7388 is dependent on wild-type p53, resistance to RG7388 could be related to *TP53* mutations that are infrequent in childhood ALL patients at diagnosis, but they increase at relapse. Since the genomic status of *TP53* gene is the major determinant of response to Mdm2 inhibitors, DNA sequencing of *TP53*, as the gold standard method, is highly recommended for identification of *TP53* mutations in primary cells, particularly in the personalized directed use of inhibitors such as RG7388[38]. It is also possible that amplification/overexpression of WIP1 in different types of cancer including hematological tumors[10] results in dysfunctional wild-type *TP53* and resistance to p53-dependent treatments comprising Mdm2 inhibitors[11].

In accord with the action mechanism of Mdm2 inhibitors, RG7388 treatment resulted in more stabilization of p53 and increased expression of its downstream targets, $p21^{WAF1}$ and Mdm2 in wild-type *TP53* Nalm6, in a concentration-dependent manner, and functional p53 primary cultures at the delivered dose of RG7388. In contrast, no significant enhancement of p53 downstream target genes was observed in mutant *TP53* CCRF-CEM and non-functional p53 ALL samples following treatment with RG7388. These outcomes are consistent with other studies that reported induction of the p53 pathway in wild-type *TP53* ALL cell lines after treatment with Nutlin-3a[36,37], CLL patients treated with RG7388[28], and Acute myeloid leukemia patients' clinical response to RG7388[39].

Given the promising phase 1 results observed in blood cancer patients treated with Mdm2 inhibitors RG73122[40], RG7388[8,39], and AMG-32[5], and the prognostic value of miR-16 expression in childhood ALL[41] and CLL[15], we evaluated the impact of miR-16-5p expression on the induction of p53 pathway and response to Mdm2 inhibitor RG7388.

Wip1 protein levels were measured at the basal level and following treatment with RG7388 in both Nalm6 and CCRF-CEM cell lines. In comparison with CCRF-CEM, in which WIP1 is highly expressed at the basal levels, WIP1 is not detectable at its basal levels in Nalm6. As expected, RG7388 treatment led to more stabilization of p53 and its target, WIP1, in a concentration-dependent manner in wild-type *TP53* Nalm6. However, there was no stabilization of p53 and no upregulation of WIP1 in *TP53* mutant CCRF-CEM cell line, demonstrating that the effect is p53-dependent. Notably, CCRF-CEM harbors a heterozygous *PPM1D* mutation (c.1327A>G; p.N443D) reported by the COSMIC (Catalogue of Somatic Mutations in Cancer)[42].

It was also shown that upregulation of miR-16-5p (by ~ 2.5-fold) significantly induced p53 stabilization (by ~ 3-fold) and upregulated its downstream target $p21^{WAF1}$ (by ~ 2.5-fold). p53 is considered as a representative haploinsufficient tumor suppressor gene in which a small change in its protein levels and/or its activity could immensely affect tumourigenesis in both mice and humans[43]. Interestingly, ectopic overexpression of miR-16-5p dramatically suppressed WIP1 expression after treatment with RG7388, followed by a significant rise in the p53 stabilization (by ~ 15.5-fold) and $p21^{WAF1}$ upregulation (by ~ 2-fold). These results were in accord with several studies indicating the therapeutic impact of combined treatment between Wip1 inhibitor, GSK2830371, and Mdm2 inhibitors for increasing sensitivity to p53-Mdm2 antagonists in wild-type *TP53* cell lines[11,31,44-46]. Mdm2 inhibitors release p53 from its negative regulator, Mdm2, which results in more stabilization of p53. Inhibition of Wip1 leads to increased phosphorylated p53 at serine 15 which activates the p53 pathway playing a critical role in cell cycle arrest mostly via upregulating of $p21^{WAF1}$, and apoptosis through upregulating proapoptotic genes including *PUMA*[11,31,46], and downregulating antiapoptotic genes particularly, *BCL2* and *BIRC5* (survivin)[46,47]. Furthermore, miR-16-5p targets multiple cell cycle genes simultaneously, which leads to the accumulation of cells in G0/G1[47-49] and directly targets the antiapoptotic *BCL2* gene which results in enhancing apoptosis[50,51] and modulating multidrug resistance. Therefore, in addition to genomic status of *TP53* and its downstream target genes involved in apoptosis and cell cycle arrest, the expression levels of miR-16-5p might be considered as a marker to predict the sensitivity to Mdm2 inhibitors like RG7388 and other drugs working through the p53 pathway[52]. It is of note that local delivery, which is limited to the localized primary tumors, systemic route, viral delivery, and non-viral administration of miRNAs, are choices in delivering miRNAs[53].

In conclusion, the current study indicated the cytotoxic effect of the Mdm2 inhibitor, RG7388, on ALL patients' primary cells in a p53-dependent manner. Moreover, it was shown that ectopic overexpression of mirR-16-5p increases sensitivity to RG7388 in ALL cells by suppressing WIP1 expression and inducing p53 stabilization. These data indicated, for the first time, the mechanistic importance of miR-16-5p in the

pathophysiology of ALL, sensitivity to RG7388, and suggested its combination with RG7388 as a novel strategy for therapeutic targeting of non-mutant p53 pediatric ALL patients.

DECLARATIONS

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Authors' contributions

Study design: Zanjirband M
Acquisition of data: Zanjirband M, Aberuyi N
Analysis and interpretation of data: Zanjirband M, Rahgozar S, Aberuyi N
Manuscript preparation: Zanjirband M, Rahgozar S, Aberuyi N
Statistical analysis: Zanjirband M, Aberuyi N

Availability of data and materials

All data generated or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study has been approved by the University of Isfahan review board and ethics committee under ethics agreement number IR.UI.1397.145. The patients' parents were clearly informed regarding the aim of the study, the method and any possible complications related to the bone marrow/blood sampling in written format and verbal explanation. Informed written consent was obtained from every patient's parent before the start of the study by trained medical staff. Participants were assured that they had the right to leave the study at their convenience. Patients' samples were identified through a code number. Clinicopathological data of patients were confidentially kept, and the electronic information was stored on a password-protected computer.

Consent for publication

Consents for publication were obtained.

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Cancer Drug Resistance

Review

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Review of 5-FU resistance mechanisms in colorectal cancer: clinical significance of attenuated on-target effects

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Abstract

The emergence of chemoresistant disease during chemotherapy with 5-Fluorouracil-based (5-FU-based) regimens is an important factor in the mortality of metastatic CRC (mCRC). The causes of 5-FU resistance are multi-factorial, and besides DNA mismatch repair deficiency (MMR-D), there are no widely accepted criteria for determining which CRC patients are not likely to be responsive to 5-FU-based therapy. Thus, there is a need to systematically understand the mechanistic basis for 5-FU treatment failure and an urgent need to develop new approaches for circumventing the major causes of 5-FU resistance. In this manuscript, we review mechanisms of 5-FU resistance with an emphasis on: (1) altered anabolic metabolism limiting the formation of the primary active metabolite Fluorodeoxyuridylate (5-Fluoro-2'-deoxyuridine-5'-O-monophosphate; FdUMP); (2) elevated expression or activity of the primary enzymatic target thymidylate synthase (TS); and (3) dysregulated programmed cell death as important causes of 5-FU resistance. Importantly, these causes of 5-FU resistance can potentially be overcome through the use of next-generation fluoropyrimidine (FP) polymers (e.g., CF10) that display reduced dependence on anabolic metabolism and more potent TS inhibitory activity.

Keywords: Fluoropyrimidine, 5-FU resistance, colorectal cancer, chemotherapy, precision medicine, thymidylate synthase

INTRODUCTION

It is estimated that 1.93 million colorectal cancer (CRC) cases will be newly diagnosed in 2022 worldwide, with 0.94 million CRC-caused deaths. According to the American Cancer Society (ACS), CRC is the 2nd most common cause of cancer-related mortality in the Unite States, accounting for ~51,000 deaths annually[1,2]. Surgical approaches are the primary treatment modality for limited-stage CRC when there is no evidence of distant metastasis. However, in elderly patients that constitute most new CRC diagnoses, there is an increased risk of post-operative complications[3]. Adjuvant chemotherapy with 5-Fluorouracil-based (5-FU-based) combinations reduces the risk of disease recurrence in stage III and high-risk stage II CRC. Chemotherapy with 5-FU-based combinations together with biologics (e.g., bevacizumab or cetuximab) and immunotherapy in some instances are also used to treat metastatic CRC (mCRC)[4,5], which often occurs in liver and is frequently not amenable to surgical resection.

The chemotherapeutic molecule most widely used for CRC treatment is 5-fluorouracil (5-FU), a synthetic fluorinated pyrimidine (FP) analog of uracil that is used to treat > 2 million cancer patients each year worldwide[6,7]. In addition to its widespread use for CRC treatment, 5-FU is also widely used to treat pancreatic, stomach, esophageal, breast, and head-and-neck cancer. 5-FU belongs to the antimetabolite class of anti-cancer drugs[8,9],{Chen, 2019 #42}{Chen, 2019 #42}{Chen, 2019 #42}{Chen, 2019 #42} and its activity results from intracellular conversion into active metabolites that interfere in thymidine biosynthesis and affect DNA- and RNA-mediated processes[10,11]{Chen, 2019 #42}{Chen, 2019 #43}. The primary molecular target of 5-FU's anti-cancer activity is thymidylate synthase (TS), which is required for *de novo* thymidylate (thymidine 5'-O-monophosphate) biosynthesis[12]. TS is a well-validated target for cancer chemotherapy[13] and aggressive malignant cells are relatively more reliant on *de novo* thymidylate biosynthesis than non-malignant cells that utilize the alternative salvage pathway[14]. The importance of targeting TS for 5-FU's anti-cancer activity is underscored by its invariant clinical use in combination with folinic acid (Leucovorin; LV), a reduced folate co-factor that binds TS in a ternary complex with 5-Fluoro-2'-deoxyuridine-5'-O-monophosphate (FdUMP), the 5-FU metabolite that irreversibly inhibits TS enzymatic activity. TS inhibition depletes cellular stores of thymidylate, resulting in increased misincorporation of 2′-deoxyuridine-5′-triphosphate (dUTP) in DNA. In cells treated with FP drugs, 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) is also misincorporated into DNA, and this causes Topoisomerase 1 (Top1)-mediated DNA damage[15]. The Gmeiner lab has developed FP polymers (e.g., CF10) that directly release FdUMP without a requirement for anabolic metabolism. CF10 inhibits TS at 100-1,000-fold lower concentrations than 5-FU in CRC cells[16-18] and causes extensive Top1-mediated DNA damage to generate increased replication stress, a point of therapeutic vulnerability in CRC cells.

While the anti-cancer activities of 5-FU and other FP drugs are considered to primarily result from TS inhibition and DNA damage, only a relatively small percentage of 5-FU administered to humans is converted to FdUMP and DNA-directed metabolites (< 5%[19]. Most 5-FU (~80%) is either degraded in the liver or excreted intact in the urine[20]. Among anabolic metabolites, ribonucleotides are produced at approximately 10-fold greater levels than deoxyribonucleotides[20,21]. The importance of RNA-directed metabolites for 5-FU's anti-cancer activity remains an active area of investigation[22]; however, the systemic toxicities associated with RNA-directed metabolites are established and include gastrointestinal tract toxicity[23,24] and immunosuppression[23,25], both of which are alleviated by uridine administration[26] to dilute

5-FU's effects on RNA-mediated processes. Patients that are deficient in 5-FU catabolism are highly vulnerable to serious systemic toxicities if treated with 5-FU[27]. Approximately 5% of the human population display polymorphisms in the gene encoding dihydropyrimidine dehydrogenase (*DPYD*) that catalyzes the initial step in 5-FU degradation and 5-FU use at standard levels is contraindicated in these patients[28].

5-FU remains a central component of CRC treatment both in the adjuvant setting and in the treatment of mCRC[4,5], which is the cause of cancer-related lethality. While 5-FU is just one component in combination therapy regimens such as FOLFOX and FOLFIRI that combine folinic acid, 5-FU, and either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), understanding the mechanistic basis for 5-FU resistance can help guide the development of new and more effective therapeutic approaches. FOLFOX or FOLFIRI are frequently used in frontline treatment of mCRC, often in combination with a biologic, such as bevacizumab[29]. While current 5-FU-based chemotherapy regimens have contributed to significantly improved survival for mCRC patients (~20 months;[30]), 5-year survival remains rare, < 14%, indicating a critical need to understand the mechanistic basis of resistance and develop new strategies to more completely eradicate metastatic disease[31]. Innate or acquired resistance remains a prominent cause of treatment failure for patients with metastatic cancer. 5-FU resistance can result from multiple causes; however, a critical review of the literature indicates cancer cells adapt to 5-FU's cytotoxic effects through: (1) decreasing intracellular FdUMP levels [Figure 1]; (2) elevating activity of the target enzyme, TS; and (3) dysregulating the balance between autophagy and apoptosis to favor cell survival. These endpoints are achieved via multiple mechanisms making overcoming resistance a challenging endeavor. This review focuses on addressing the causes of clinical resistance to 5-FU, considering both clinical data and cellular models of CRC. We review mechanisms by which 5-FU-based therapy fails, intending to provide insight into novel strategies to overcome resistance and improve outcomes beyond the incremental gains achieved in recent years[32].

CLINICAL DETERMINANTS OF 5-FU RESPONSE IN CRC TREATMENT

The applicability of 5-FU-based chemotherapy for CRC treatment depends upon several factors. For patients with stage III CRC or diagnosed with stage II CRC with risk factors consistent with an elevated likelihood for relapse, 5-FU-based adjuvant chemotherapy is recommended unless tumor biopsy demonstrates high microsatellite instability (MSI-H) or deficiency in DNA mismatch repair (MMR-D). For patients with MSI-High or MMR-D primary CRC tumors, which include familial syndromes such as Lynch syndrome, 5-FU-based regimens are ineffective and testing for MMR-D status prior to treatment is standard care. Testing for MMR-D status is also required for establishing responsiveness to immune checkpoint blockade immunotherapy, which is relatively more effective in CRC patients with high tumor mutational burden associated with MMR-D[33]. MSI testing by polymerase chain reaction (PCR) and immunohistochemistry (IHC) is used to establish MMR-D[34]. Two antibody IHC testing for MSH6 and PMS2 is used to identify MMR-proficient CRC patients, and if deficiency is suspected, IHC for mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) are undertaken to establish MMR-D[35]. MLH1 promoter methylation testing is done for cases with MLH1-IHC loss.

Relevance of MMR-D for CRC chemotherapy is that, in general, MSI-High and MMR-D in early-stage primary colon cancer confer a good prognosis and NCCN does not recommend adjuvant 5-FU for stage II CRC that is MSI-high. However, the FOLFOX regimen is beneficial in MSI-high stage III[36], and patients with Transforming growth factor-β_{RII} (TGF-β_{RII}) mutations in particular may be responsive to 5-FU-based therapy[37]. The TGF-β pathway also is implicated in drug resistance in pre-clinical studies and specific inhibition of TGF-β_I restored the sensitivity of resistant CRC cells to 5-FU[38]. Similarly, for patients with mCRC that is MSI-H or MMR-D, alternative frontline therapy to 5-FU-based therapy is implemented,

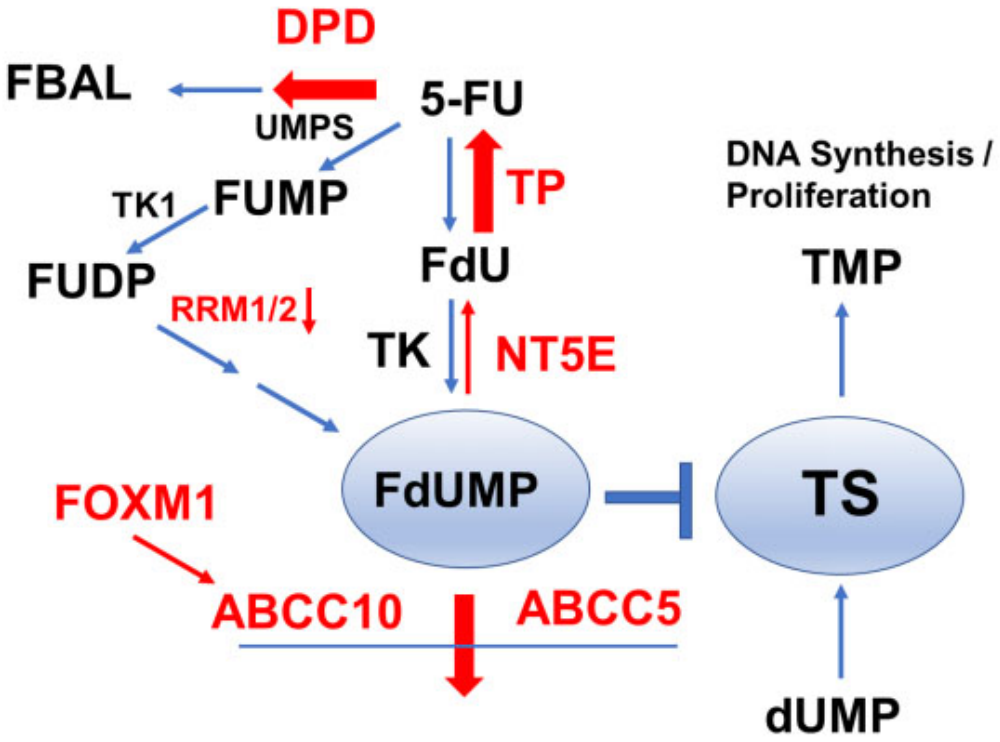

Figure 1. 5-FU Resistance can develop through either increased intratumor degradation, decreased anabolic metabolism to FdUMP and/or increased efflux of FdUMP - all of which decrease TS inhibition in tumor cells. 5-FU may be degraded via intratumor DPD, while its anabolic metabolism to FdUMP occurs primarily via UMPS/RRM1/2. Decreased FdUMP levels may result from less efficient conversion through decreased RRM1/2 expression or from increased FdUMP breakdown through elevated expression of NT5E and TP. FdUMP also undergoes efflux from tumor cells by ABC transporters, ABCC10 and ABCC5, that are upregulated upon increased FOXM1 expression in 5-FU-resistant cancer cells. Processes contributing to decreased intra-tumor FdUMP and 5-FU resistance are indicated by red arrows. 5-FU: 5-Fluorouracil; FdUMP: 5-Fluoro-2′-deoxyuridine-5′-O-monophosphate; UMPS: UMP synthase; FBAL: α-fluoro-β-alanine; DPD: dihydropyrimidine Dehydrogenase Deficiency; FUMP: 5-fluorouridine-5'-monophosphate; FUDP: 5-fluorouridine diphospho; FdU: 5-fluoro-2′-deoxyuridine; TK: thymidine kinase; TS: thymidylate synthase; TMP: Trimethylolpropane; RRM: RNA recognition motif; NT5E: ecto-5′-nucleotidase; FOXM1: forkhead box M1; dUMP: 2′-deoxyuridine-5′-O-monophosphate.

frequently immune checkpoint blockade[39]. The mechanism by which deficient MMR renders 5-FU-based regimens ineffective is not definitively known. In principle, MMR may remove 5-FU from DNA and genomic silencing or mutation of MMR genes may increase 5-FU in DNA[40], which could accentuate DNA damaging processes, which include DNA topoisomerase 1 poisoning[41]. Alternatively, MMR proficiency may contribute to cancer cell death through the activation of a futile cycling mechanism[42]. Our studies indicate MMR status does not significantly affect the response of CRC cells to either 5-FU or CF10[43], indicating the observed clinical cause of MMR dependence may not be directly related to DNA repair. Base excision repair (BER) actively removes 5-FU from DNA in CRC cells[44,45], but BER is not a determinant in 5-FU clinical response. However, CpG island methylator phenotype, and chromosome instability[46], two processes that differentiate the etiology of CRC development, are implicated in 5-FU response[47,48].

MECHANISMS INCREASING TS TO CAUSE 5-FU RESISTANCE

TS is considered the primary molecular target for the anti-cancer activities of FP drugs (5-FU, capecitabine, and floxuridine). TS also is targeted by anti-folates such as tomudex and raltitrexed[49]. The clinical success of these drugs, as well as more recent FP-based combination approaches that target TS such as S1 (tegafur, gimeracil, potassium oxanate)[50] and Lonsurf or TAS-102 (trifluridine, Tiperacil)[51], establishes TS as a

central and well-established chemotherapy target[13]. The structural basis for TS inhibition by FPs was shown to result from nucleophilic attack by Cys195 at C6 of FdUMP, resulting in irreversible enzyme inhibition via a ternary complex that also includes a reduced folate co-factor[49].

The relationship between TS levels and response to 5-FU, other FPs, or TS inhibitors is complex, in part because while elevated TS levels contribute to resistance (since more FdUMP is required for TS inhibition), but very low TS levels slow cell proliferation, which is necessary for replication-dependent DNA damage. Further, establishing elevated TS as a cause of resistance to 5-FU or other TS-targeted therapeutics is challenging because TS is regulated at multiple levels including through gene amplification, polymorphisms in the promoter, and upregulation of transcription factors that regulate its intratumor expression [Figure 2]. TS levels and activity[52] significantly correlate with response to 5-FU-based treatment and LV enhanced TS inhibition. However, incorporation of 5-FU into either DNA or RNA does not correlate with response to 5-FU[53].

Transcriptional regulation of TYMS

Transcriptionally, *TYMS* (encoding TS) is regulated by E2F family transcription factors[54] in an S-phase-dependent manner[55]. *TYMS* expression is also sensitive to Myc levels and silencing *TYMS* decreases the oncogenic properties of elevated MYC in some cell contexts[56]. Recently, an analysis from the Cancer Genome Atlas (TCGA) database revealed lower *TYMS* was associated with better response to FOLFOX/FOLFIRI therapy in mCRC patients and MYC was identified as an upstream controller of genes that regulate response to 5-FU+folate therapy[57]. The forkhead transcription factor forkhead box M1 (FOXM1) is regulated by E2F1 and directly upregulates *TYMS* and is responsive to DNA damage. Elevated FOXM1 is a cause of 5-FU resistance through the upregulation of *TYMS*[58], and recent studies indicate targeting FOXM1 can overcome 5-FU resistance[59]. Other signaling pathways may upregulate *TYMS* and cause 5-FU resistance, including HSP90/Src[60]. Further, *TYMS* is regulated by the MALAT1-miRNA network[61] and other miRNAs that regulate drug resistance[62] and can be used as biomarkers[63].

Gene amplification of TYMS

The importance of TS gene and protein expression for 5-FU resistance was established in CRC tumors. Responsive patients had significantly lower mean TS protein and gene levels relative to non-responsive patients[64]. Further, CRC cells selected for acquired 5-FU resistance displayed elevated TS, which occurred through gene amplification[65]. Elevated TS is associated with clinical resistance to 5-FU[66], consistent with TS being the primary molecular target of FPs. Several studies[67,68], including a meta-analysis of 13 studies[69], demonstrated that elevated TS was associated with poor outcomes. However, multiple studies indicate the relationship between TS expression and 5-FU response is complex and may depend on the extent of TS nuclear localization or the expression of other genes, particularly those regulating 5-FU metabolism including dihydropyrimidine dehydrogenase deficiency (DPD) and TP[66]. TS undergoes reversible SUMOylation[70] and localizes to the nucleus (nTS) as part of a multi-protein complex that enables efficient *de novo* ddP biosynthesis during S-phase[71]. Clinical studies indicate that increased intratumor nuclear localization of TS may be a better indicator of disease aggressiveness than overall TS levels[72]. *TYMS* gene amplification is detected in mCRC from patients pre-treated with 5-FU-based chemotherapy and was associated with shorter median survival for patients treated with chemotherapy following surgical resection[73]. A summary of studies in which TS gene amplification was implicated with 5-FU resistance is included in Table 1.

TSER and alternative causes of elevated TS

In addition to *TYMS* gene amplification and increased transcription, at least three other processes are potential factors that could increase TS levels and contribute to 5-FU resistance [Figure 2]: i) TS enhancer

Table 1. TS Gene Amplification in 5-FU Resistance

Tissue/cells	Frequency/treatment	Site	Reference
mCRC	18%	Liver metastases	[73]
mCRC	23%	Liver metastases	[74]
CRC	Increased progression	Colon cancer	[75]
CRC cells	FdU treatment	Colon cancer cells	[76]
CRC cells	5-FU treatment	Colon cancer cells	[65]

TS: thymidylate synthase; mCRC: metastatic colorectal cancer; CRC: colorectal cancer.

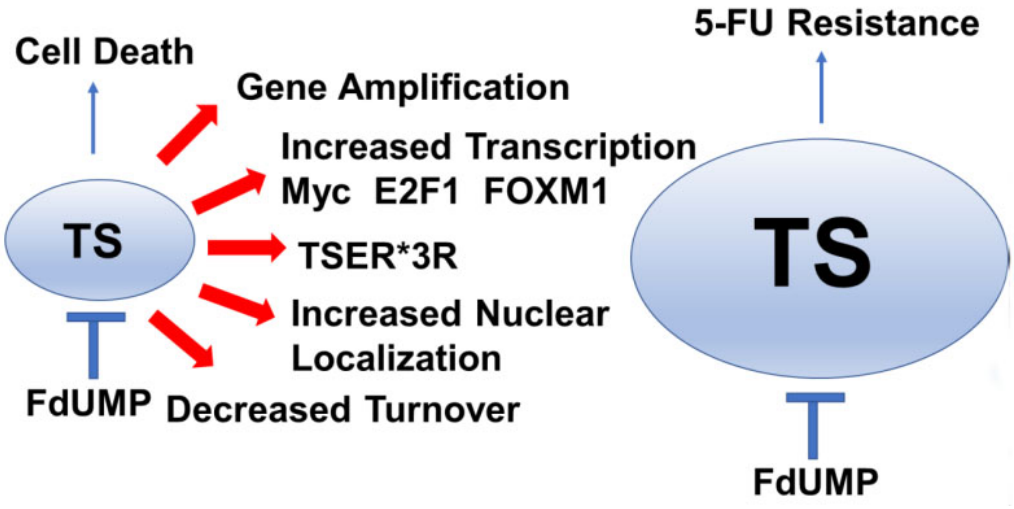

Figure 2. 5-FU Resistance develops from processes that increase thymidylate synthase (TS) activity in cancer cells. Increased TS activity can result from multiple processes including gene amplification, increased transcription, TSER*3R polymorphism, increased TS nuclear localization, and decreased TS protein degradation, which are indicated by red arrows. Increased TS activity renders cells 5-FU-resistant because FdUMP levels are insufficient to inhibit all the TS available. 5-FU: 5-Fluorouracil; FdUMP: 5-Fluoro-2′-deoxyuridine-5′-O-monophosphate; FOXM1: forkhead box M1.

region (TSER) polymorphisms; ii) TS translational autoregulation; iii) TS proteasomal degradation. Polymorphisms in the 5′-UTR of *TYMS* contribute to elevated *TYMS* expression in some contexts[77,78]. A triple tandem repeat (TSER*3)[79] in the 5′-UTR of the TS gene[80] resulted in elevated *TYMS* expression and 5-FU resistance[81]. The clinical significance of TSER genotypes remains largely unproven in CRC; however, prospective selection of patients with gastric cancer have at least one TSER*2 allele favoring lower *TYMS* expression therapy resulted in an encouraging disease control rate for treatment with FOLFOX[82]. Further, *TYMS* polymorphisms, together with *KRAS* and *BRAF* mutation status, retrospectively, were associated with reduced relapse in CRC[83]. TS also poses a negative autoregulatory function at the translational level by binding to its own mRNA; thus, it prevents the synthesis of functional TS enzyme[73]. Autoinhibition of TS protein expression is countered by FdUMP binding and ternary complex formation. At present, there is no evidence that autoregulation of TS by this mechanism contributes to 5-FU resistance. However, another aspect of translation is affected by 5-FU, which is the efficiency and selection of proteins translated by the ribosome[84]. TS also undergoes proteasomal degradation and TS levels reflect a dynamic balance of new protein synthesis, dependent upon gene expression and translational efficiency, that is countered by the rate of degradation for expressed protein. A recent study showed that decreased O-GlcNAc transferase (OGT), an enzyme responsible for post-translational modification of multiple proteins including TS, affected TS proteasomal degradation in 5-FU-resistant cells[85].

Increased TS and intrinsic 5-FU resistance

The elevated expression of TS is commonly accepted as a primary molecular mechanism for acquired 5-FU resistance[86], but it also is important for intrinsic resistance. The stability of the ternary complex is highly dependent on 5,10-methylenetetrahydrofolate (CH_2THF) levels[78], and lack of CH_2THF creates an unstable TS: FdUMP binary complex resulting in poor inhibition[81,86,87]. Increased TS level prior to 5-FU-based treatments is associated with perturbed folate pools, which cause intrinsic resistance compared to acquired resistance associated with upregulated *TYMS* expression and gene amplification[73,86]. These findings suggest that patients with tumors showing TS amplification prior to treatment should not be treated with 5-FU to avoid systemic toxicity without the likelihood of clinical benefit[73,74].

GENES MODULATING 5-FU METABOLISM

Acquired drug resistance is a principal cause of treatment failure and significantly contributes to cancer-related mortality. In the case of 5-FU, elevated TS is clinically established as a significant cause of drug resistance[69]. Still, other reasons have been identified, and prominent among them are alterations in genes that modulate 5-FU metabolism, affecting both its degradation and its conversion to FdUMP, the TS inhibitory metabolite[88] [Figure 1]. A key aspect of 5-FU activity, toxicity, and resistance is mediated by *DPYD*, the gene encoding DPD, the first and rate-limiting step in 5-FU degradation. Atypical 5-FU degradation in liver is associated with serious systemic toxicities[89]. In many countries, genetic screening is used to identify CRC patients with *DPYD* polymorphisms associated with decreased DPD activity that result in serious 5-FU toxicities unless the administered dose is reduced from standard dosing[90]. Since DPD is not the only potential cause of altered 5-FU toxicity or sub-optimal therapeutic response, alternative procedures such as therapeutic drug monitoring[91] are used to quantify patient response on an individualized basis and to customize 5-FU treatment to account for individual variations in drug metabolism.

Intratumor 5-FU catabolism

In addition to the role of *DPYD* polymorphisms in modulating 5-FU toxicity and therapeutic response by affecting systemic drug degradation, intra-tumoral *DPYD* expression is an important factor in modulating therapeutic response. For example, elevated intra-tumoral *DPYD* expression, together with elevated *TYMS*, is associated with poor outcomes in CRC patients treated with 5-FU-based chemotherapy[66]. A third gene, thymidine phosphorylase (TP; encoded by *TYMP*), was implicated together with *DPYD* and *TYMS* in this study. TP catalyzes a reversible reaction that may produce thymidine or 2'-deoxyuridine, or analogs such as 5-fluoro-2'-deoxyuridine (FdU), from their respective nucleobases (e.g., 5-FU), together with 2'-deoxyribose 1-phosphate. Alternatively, TP degrades thymidine analogs such as FdU to the nucleobase after dephosphorylation by ecto-5′-nucleotidase (NT5E)[92]. The directionality of TP catalysis depends on intra-tumor substrate/product ratios; however, levels of 2'-deoxyribose 1-phosphate in plasma were also found to be predictive of chemotherapy sensitivity in gastric cancer that included a fluoropyrimidine[93]. Findings from this study[66] that elevated *TYMP* levels together with *TYMS* and *DPYD* are associated with decreased response to 5-FU are consistent with TP primarily catalyzing FdU degradation in CRC tumors and resistance to 5-FU is associated with elevated *TYMP* expression. Further, TP-mediated degradation of trifluorothymidine (TFT), the FP component of TAS-102, limits activity resulting in the inclusion of a TP inhibitor, Tipiracil[94]. TP is also known as platelet-derived endothelial cell growth factor (PDECGF), a growth factor promoting angiogenesis, and increased PDECGF/TP is a prognostic factor for poor survival in CRC[95] that acts through the production of 2'-deoxyribose 1-phosphate from thymidine to promote chemotaxis of vascular endothelial cells[96].

Anabolic 5-FU metabolism and resistance

The anabolic biosynthesis of FdUMP from 5-FU can occur via either of two major pathways: (1) TP/thymidine kinase (TK) in which FdUMP is produced by 5-FU in two steps; or (2) via a multi-step biosynthetic pathway (UMPS/RNR) that involves UMP synthase (UMPS), uridine kinase (UK), and UMP kinase (UMPK) to produce FUDP. FUDP is a substrate for ribonucleotide reductase (RNR) to produce FdUDP, which can be converted to 5-fluoro-2'-deoxyuridine-5'-diphosphate (FdUDP) through conversion to FdUTP followed by dUTPase cleavage. Enzymes important for the *de novo* biosynthesis of pyrimidines are upregulated in CRC relative to non-malignant tissue[97], and reduced activities of these enzymes which may occur via altered splicing[88,98] are associated with 5-FU resistance[99]. The importance of FdUMP biosynthesis via the UMPS/RNR pathway is demonstrated by studies that identify reduced expression and activity of enzymes in this pathway in 5-FU-resistant cells. Studies in KM12C xenograft tumors showed resistance to 5-FU was associated with decreased RNR activity[100], while analysis of clinical samples indicated 5-FU resistance was associated with high TS mRNA and low RNR activity[101].

Collectively, the preponderance of evidence indicates that altered *de novo* thymidine biosynthesis, either by affecting TS expression [Figure 2] or modulating genes important for 5-FU anabolic metabolism to FdUMP [Figure 1], is central to 5-FU resistance. In a few instances, 5-FU resistance is mediated by changes affecting RNA-directed processes including tRNA modifications[102,103] and rRNA[22]. However, the clinical significance of RNA-directed activities for 5-FU anti-tumor activity is not yet proven. Further evidence for anabolic metabolism of 5-FU to FdUMP being important for 5-FU resistance comes from studies demonstrating elevated expression of ABCC10[104] and ABCC5[105], two ATP binding cassette proteins[106] that mediate FdUMP efflux from 5-FU-treated cells, cause of 5-FU resistance as does elevated FOXM1, a major transcriptional regulator of ABCC10[104] [Figure 1].

CELL DEATH SIGNALING IN 5-FU RESISTANCE

The cytotoxicity of multiple anti-cancer drugs, including 5-FU, depends on the activation of programmed cell death that irreversibly commits drug-treated cells to destruction[107,108]. p53 is considered to be the most highly mutated gene in cancer and it plays a central role in determining if drug-treated cells undergo cell cycle arrest mediated by p53's downstream effector p21, or initiate apoptosis mediated by Bax and other p53-dependent pro-apoptotic genes[109] [Figure 3]. In the case of established DNA-damaging drugs such as Adriamycin, either p53 or p21 deficiency leads to loss of the G1/S checkpoint and efficient apoptosis[110]. However, 5-FU deletion of p53 in HCT-116 cells resulted in resistance to apoptosis and 5-FU was less effective towards $p53^{-/-}$ HCT-116 xenografts relative to isogenic tumors that were $p53^{+/+}$. Furthermore, 5-FU-induced apoptosis both required p53 and was inhibited by exogenous uridine, but not thymidine, consistent with apoptosis induction in response to an RNA-directed process under these treatment conditions[110]. Studies from our laboratory confirm that p53 deletion causes 5-FU resistance in HCT-116 cells with expression of the R248W gain of function p53 mutation causing greater resistance, while the DNA-directed FP polymer CF10 showed reduced resistance indices relative to 5-FU[1].

However, even in cell models of CRC, there is variability in the extent that p53 is required for 5-FU-induced apoptosis. Studies report that 5-FU-induced apoptosis occurs in both wild-type and mutant p53 CRC cells with increased expression of the pro-apoptotic Bcl-2 family proteins Bax and Bak identified as being particularly important for 5-FU-induced apoptosis[111] [Figure 3]. The importance of p53 for regulating 5-FU-induced apoptosis has also been shown to occur via altered chromatin accessibility upon 5-FU treatment that affects the transcription of genes important for apoptosis[112]. The clinical significance of p53 mutations for 5-FU resistance is not established, although some clinical data indicate *TP53* mutations confer a worse prognosis[113], while p53 together with Rb and the anti-apoptotic bcl-family member Mcl-1

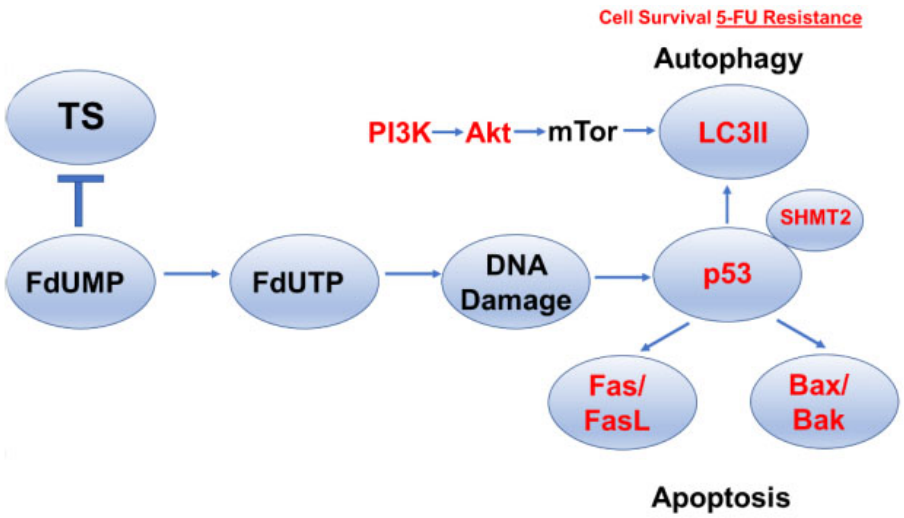

Figure 3. 5-FU Resistance develops from an altered balance between autophagy, which favors cell survival and apoptosis in 5-FU-treated cells. p53 is a key regulator of autophagy/apoptosis balance in 5-FU-treated cells and is modulated by SHMT2. Increased signaling through the PI3K/Akt/mTOR pathway stimulates LC3II and upregulates autophagy to promote cell survival and 5-FU resistance. Induction of apoptosis involves upregulation of the death receptor pathway or mitochondrial pathway for programmed cell death, and downregulation of Fas/FasL or Bax/Bak decreases 5-FU-induced apoptosis and promotes cell survival and 5-FU resistance. 5-FU: 5-Fluorouracil; TS: thymidylate synthase; FdUMP: 5-Fluoro-2′-deoxyuridine-5′-O-monophosphate; FdUTP: 5-fluoro-2′-deoxyuridine-5′-triphosphate; SHMT2: serine hydroxymethyl transferase.

correlated with clinical outcomes in patients with colorectal liver metastases[114]. Factors other than p53 that are important for activating apoptosis in response to 5-FU treatment include Fas, which was shown to be induced in response to 5-FU treatment in a p53-dependent manner and to regulate apoptosis[115] [Figure 3]. Houghton and co-workers showed that different CRC cell lines varied in the efficiency of Fas upregulation in response to 5-FU/LV treatment and this correlated with the efficiency of thymidine reversal, indicating 5-FU's DNA-directed effects were cell line dependent and correlated with Fas upregulation and activation of extrinsic apoptosis[116]. Resistance to 5-FU-induced Fas upregulation and apoptosis can occur via methylation of the Fas promoter, silencing its expression, which can be reversed by 5-Aza deoxycytidine[117]. Clinical significance for Fas upregulation in 5-FU response was shown by increased Fas expression in biopsy specimens following 5-FU treatment[118].

AUTOPHAGY IN 5-FU RESISTANCE

Autophagy plays an important role in tumorigenesis and modulating drug response and can either be complementary to apoptosis by promoting drug lethality or protective of the cytotoxic effects of drug treatment[119]. Activation of the PI3K/Akt/mTOR signaling pathway upregulates autophagosome formation and inhibitors of this pathway modulate drug response, in part, through downregulating autophagy. Treatment of CRC cells with 5-FU was shown to increase LC3-II levels consistent with autophagy activation and co-treatment with 3-methyl adenine (3-MA), a PI3K inhibitor, blocked autophagosome formation and promoted 5-FU-induced apoptosis implicating a protective role for autophagy in 5-FU treatment[120] [Figure 3]. However, reduced autophagy has also been reported in 5-FU-resistant CRC cells[121]. Overactivation of the Akt pathway is also associated with 5-FU resistance and Akt inhibition may overcome resistance in some CRC cells[122], in part by modulating autophagy, while miRNA regulation of Akt deactivation by the PP2A phosphatase complex also regulates 5-FU resistance[123]. The autophagy inhibitor chloroquine also enhanced the lethality of 5-FU to CRC cells, and in these studies, a serine hydroxymethyl transferase (SHMT2) was shown to regulate 5-FU resistance by binding p53 and inhibiting its degradation [Figure 3]. Overall, SHMT2 was upregulated in CRC tissue compared to non-malignant tissue; however,

patients with low SHMT2 had worse outcomes and this correlated with elevated LC3-II and p62 consistent with autophagy activation in SHMT2-low, 5-FU-resistant CRC[124]. Interestingly, trifluorothymidine (TFT), the FP used in TAS-102, differed from 5-FU in the extent of activating autophagic survival[125]. Activation of the p38MAPK pathway is also a determinant in autophagy activation and modulates cellular responses to 5-FU. Inhibition of the p38MAPK pathway correlated with attenuation in 5-FU-mediated apoptosis and promoted CRC cell resistance[126]. Thus, the p38MAPK signaling pathway modulates 5-FU resistance by regulating the pivot between autophagy and apoptosis[126]. The autophagy-regulated gene HSPB8 was found to be key in regulating interactions with the tumor microenvironment that regulate 5-FU resistance[127]. Autophagosome formation is also regulated by Rho kinases[128], which are implicated in 5-FU resistance[129]. Curcumin has been studied to inhibit AMPK/ULK1-dependent autophagy with the potential to overcome 5-FU resistance through autophagy activation[130], and studies from our laboratory indicate curcumin enhances the cytotoxicity of DNA-directed polymeric fluoropyrimidines[131,132].

CONCLUSION

5-FU remains central to the management of colorectal cancer and it is widely used both in the adjuvant setting to treat CRC patients with limited-stage disease and in combination with chemotherapy regimens to treat mCRC[4]. The evasion of 5-FU cytotoxicity through intrinsic or acquired resistance in patient tumors contributes to poor outcomes manifest either as a high rate of relapse despite adjuvant chemotherapy or limited survival in the metastatic setting despite multiple lines of chemotherapy that include 5-FU or other FP drugs[5]. Lack of response to 5-FU chemotherapy is predictable in patients with deficiencies in DNA MMR, or high microsatellite instability, and therapy with 5-FU is contra-indicated in these patients. 5-FU therapy is also predictably toxic in patients with polymorphisms in *DPYD* that limit 5-FU degradation in the liver[89], and these patients require special management[90]. Beyond these limited exclusions, there are currently no defined criteria for determining which CRC patients are not likely to be responsive to 5-FU-based therapy. Thus, there is a need to systematically understand the mechanistic basis for 5-FU treatment failure, and an urgent need to develop new approaches for circumventing major causes of 5-FU resistance.

In this review, we have summarized major mechanisms that contribute to 5-FU resistance with an emphasis on those for which available data support clinical significance and that affect the on-target activity of 5-FU (TS inhibition). The causes of colorectal cancer are multi-factorial and involve both lifestyle choice and personalized genetic susceptibility[133]. Further, response to treatment also depends on multiple factors[134]. Collectively, the reviewed literature consistently implicates resistance as developing from processes that limit the anabolic metabolism of 5-FU to FdUMP, the TS inhibitory metabolite [Figure 1], and from mechanisms that result in elevated TS activity that results from gene amplification, polymorphisms in the TS promoter, elevated levels of transcription factors that regulate *TYMS* expression, and/or altered nuclear localization of TS [Figure 2]. Dysregulation in the balance between cell survival and programmed cell death is also important in the development of 5-FU resistance [Figure 3]. We have not attempted to review miRNA regulation of *TYMS* [61] or other epigenetic causes of 5-FU resistance[135], and these have recently been reviewed[136-138]. We also have not directed the reader to literature focused on cellular changes that promote quiescence and stemness to escape the cytotoxic effects of 5-FU[139,140], although these are likely to be of clinical significance. Further, it is clear that the tumor microenvironment modulates therapy response by processes independent of on-target effects on cancer cells and these processes are not reviewed in this manuscript.

The focus of this review is on acquired resistance to 5-FU with decreased anabolic metabolism and elevated TS activity[49] as clinically relevant causes. In principle, these causes of 5-FU can be addressed through the translation of next-generation FP drugs that retain the anti-tumor activity associated with targeting TS in

CRC, but that do not require multiple steps of anabolic metabolism required by 5-FU. TAS-102, a combination of the FP trifluorothymidine and a TP inhibitor Tiperacil, shows efficacy in 5-FU-resistant models and activity in refractory metastatic CRC[141]. TAS-102 activation requires thymidine kinase and it is not a substrate for DPD[94]. Our laboratory has pioneered the development of DNA-based FP polymers to deliver Fluorodeoxyuridylate, the TS-inhibitory metabolite of 5-FU, without a requirement for metabolic activation. We showed that the prototype FP polymer F10 was, on average, 338-fold more potent than 5-FU across the NCI60 cell line screen[15,135], but it was still very well tolerated *in vivo*[142], indicating 5-FU toxicities do not necessarily arise predominantly from on-target effects. The 2nd generation FP polymer CF10 is even more potent and shows promising activity in pre-clinical models of CRC and pancreatic cancer[16,17]. Further, the cytotoxic mechanism of CF10 results from both inhibiting TS and poisoning of DNA topoisomerase 1 (Top1)[41,143], which results from a distinct mechanism distinct from current Top1 poisons in clinical use[144]. In summary, the next generation of FPs has the potential to overcome the established mechanism of resistance for 5-FU reviewed herein that has limited clinical response to FPs to date.

DECLARATIONS

Authors' contributions

Conceptualization: Gmeiner WH, Okechkwu CC
Original draft preparation: Okechkwu CC, Gmeiner WH
Writing, review, editing, visualization, supervision, and funding acquisition: Gmeiner WH
Both authors have read and agreed to the published version of the manuscript.

Availability of data and material

Not applicable.

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Conflicts of interest

Gmeiner WH is an inventor on a pending patent application on CF10 for the treatment of colorectal cancer.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Opinion

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Mitochondria in colorectal cancer stem cells - a target in drug resistance

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Abstract

Colorectal cancer (CRC) is the third most diagnosed cancer and the second most deadly type of cancer worldwide. In late diagnosis, CRC can resist therapy regimens in which cancer stem cells (CSCs) are intimately related. CSCs are a subpopulation of tumor cells responsible for tumor initiation and maintenance, metastasis, and resistance to conventional treatments. In this scenario, colorectal cancer stem cells (CCSCs) are considered an important key for therapeutic failure and resistance. In its turn, mitochondria is an organelle involved in many mechanisms in cancer, including chemoresistance of cytotoxic drugs due to alterations in mitochondrial metabolism, apoptosis, dynamics, and mitophagy. Therefore, it is crucial to understand the mitochondrial role in CCSCs regarding CRC drug resistance. It has been shown that enhanced anti-apoptotic protein expression, mitophagy rate, and addiction to oxidative phosphorylation are the major strategies developed by CCSCs to avoid drug insults. Thus, new mitochondria-targeted drug approaches must be explored to mitigate CRC chemoresistance via the ablation of CCSCs.

Keywords: Cancer stem cells, mitochondria, colorectal cancer, drug resistance, mitophagy

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer worldwide, the second leading cause of cancer death in women, and the third in men[1]. Currently, the chemotherapy regimens for CRC consist of 5-fluorouracil (5-FU) plus leucovorin for early stages (stages 0, I, and II), and 5-FU plus oxaliplatin (FOLFOX) or 5-FU plus irinotecan (FOLFIRI), which may be accompanied by targeted therapies (e.g., bevacizumab or cetuximab), as a first-line treatment for more advanced stages (stages III and IV)[2]. FOLFOX and FOLFIRI chemotherapy regimens achieve objective response rates of approximately 50% and the overall CRC patients present 5-year survival rates of 60%. Although there are tools for genetic screening of CRC for defective DNA mismatch repair (MMR) using immunohistochemistry and/or microsatellite instability test[3], diagnosis of disease at a late stage (stage IV) is frequent in CRC, which reduces the overall 5-year survival rate to approximately 18%[1]. In addition, nowadays, it is known that miRNAs play an important role in resistance to the chemotherapy regimens mentioned above, which makes them a relevant target for major studies[4].

Regarding therapy resistance, researchers have sought new strategies, such as using CRC patient-derived organoids (PDOs), to understand this challenging problem. Recent studies suggest that PDOs can prevent patients from undergoing ineffective chemotherapy and can help to develop effective and personalized therapies against CRC[5,6]. However, drug resistance is still a problem to be fought, with cancer stem cells (CSCs) emerging as an important factor in the process[7]. CSCs are a subset of tumor cells defined by their ability to self-renewal and differentiate into distinct progenies, being also responsible for invasion and migration, leading to metastasis, and a slow-cycling cellular turnover, making them resistant to therapies[8,9]. CSCs have been described in many types of cancer, and colorectal cancer stem cells (CCSCs) are considered the major cause of therapeutic failure and resistance to CRC treatments, leading to tumor progression, recurrence, and, eventually, patient death[10].

The mechanisms underlying CCSCs chemoresistant phenotype include reversible quiescent state, high expression of drug efflux pumps, activation of protumoral signaling pathways, such as Hedgehog, Notch, Wnt/β-catenin, Hippo, PI3K/Akt, and TGF-β, avoidance of chemotherapeutic-induced DNA damage, dysregulation of microRNAs, hypoxia microenvironment, metabolic switch for oxidative phosphorylation (OXPHOS), among others[2].

Since conventional anticancer therapies act on highly proliferating cells, it does not affect quiescent cancer stem cells, as they are restrained in the G0 phase from the cell cycle, a non-proliferative state. Additionally, these cells can re-enter the cell cycle after undergoing chemotherapy, leading to cell proliferation and tumor regeneration[11]. Francescangeli *et al.*[12] showed a high proportion of quiescent and chemoresistant CCSCs in response to treatment with oxaliplatin and 5-fluorouracil in a xenograft model.

Several studies have shown that mitochondria are involved in the mechanisms of chemoresistance of cytotoxic drugs or radiotherapy due to the many metabolic pathways conferring tumor cells' resistant phenotype and, specifically, CSCs[13-15]. Therefore, it is important to understand the mitochondrial role in CRC drug resistance mediated by cancer stem cells.

MITOCHONDRIAL ROLE IN DRUG RESISTANCE: AN OVERVIEW

Beyond the powerhouse of the cell, mitochondria have been shown to act in many different frontlines, such as cell survival, proliferation, autophagy, and calcium homeostasis[16]. In cancer, these double-membrane organelles play a pivotal role in establishing and progressing the disease, ranging from mitochondrial dynamics, biogenesis metabolism, and cell death regulation. Reactive oxygen species (ROS) balance

mitochondria DNA (mtDNA) mutations[17], drug resistance, and mitochondria work like stress sensors, mediating adaptations in the face of an adverse environment caused by chemo or radiotherapies[18,19].

Some major mechanisms for drug resistance include alterations in drug transport and metabolism, enhanced DNA damage repair machinery, and inhibition of apoptosis[20]. Most chemoresistant tumors present a high expression of drug efflux membrane transporters, wherein the ATP-binding cassette (ABC) transporter family is the most important, highlighting ABCB1 and ABCG2, which are key players in cancer chemoresistance as they are capable of transporting the majority of conventional chemotherapeutic agents[21] Since ABC transporters depend on ATP hydrolysis to pump chemotherapeutics, mitochondria have their importance heightened as they are major producers of ATP via OXPHOS. A recent study using chemoresistant ovarian cancer cells showed that electron transport chain inhibitors increased drug retention and reverted the chemoresistant phenotype in vitro and *in vivo*[22].

Mitochondria morphology is also an important feature of drug resistance. It is a dynamic organelle and can vary in size and copies inside cells due to fusion and fission, which are regulated by a GTPase family of proteins. Mitochondrial fusion, which usually occurs in nutrient deprivation and increased OXPHOS cases, implicates long interconnected tubules, forming networks. Oppositely, mitochondrial fission appears as small and fragmented mitochondria, commonly associated with cellular and mitochondrial dysfunction, severe stress, and increased proliferation[23]. A study using cisplatin-resistant cervical and ovarian cancer cell lines exhibited a prevalence of elongated mitochondria, similar to tubular shapes, suggesting that mitochondria fusion is required to resist the drug treatment[24]. Nevertheless, studies in breast cancer and acute lymphoblastic leukemia cellular lineages have demonstrated that mitochondria fission is required for metabolic adaptation favorable for protecting cells from chemotherapy agents[25,26]. In addition, mitochondrial fission factor (MFF) is upregulated in prostate cancer stem cells compared to the primary tumor and normal prostate cells[27]. A novel study with metastatic breast cancer cells shows that changes in mitochondrial dynamics, tending to a fission increase, lead to increased production of mitochondrial ROS, which retrograde response endows cells with resistance to oxidative stress and reactive oxygen species-dependent chemotherapy drugs[28]. Therefore, the role of mitochondrial dynamics on drug resistance is an important area for further study.

Considering that mitochondria act as a metabolic hub, these organelles are expected to have great importance in modulating therapy sensitivity, especially by controlling redox metabolism[29]. Resistant cancer cells usually exhibit great mitochondrial functionality, with high rates of OXPHOS. For instance, many studies have shown that OXPHOS inhibitors suppress resistance to anticancer drugs in lung adenocarcinoma, prostate cancer, melanoma, pancreatic cancer, and colon cancer[30]. A study showed that treating ovarian cancer cells with cisplatin might induce oxidative phosphorylation and stem cell enrichment, which can be overcome using OXPHOS inhibitors[31]. Fatty acids oxidation (FAO), which depends on mitochondrial enzymes, is also considered an accomplice of therapeutic resistance[32], as a study demonstrated that FAO inhibition promotes chemosensitivity in breast CSCs[33]. Lee *et al.*[34] showed that liver cancer stems cell chemoresistance depends on glutamine metabolism by mitochondria since mitochondrial ATP used in drug efflux is provided by glutamine, and its inhibition reduces efflux, decreasing chemoresistance. Increased expression of MTHFD2, a mitochondrial enzyme involved in the one-carbon metabolic pathway, was observed in gefitinib-resistant lung cancer cells with stem cell features. MTHFD2 knockdown decreased stem cell phenotype and promoted gefitinib sensitization, highlighting the MTHFD2 importance and potential as a target for anticancer therapy[35]. Another important pathway in which mitochondria participate in is folate metabolism, also known as 1C metabolism[36]. This metabolic process involves the activation and transference of one carbon-molecules to support nitrogenous bases

biosynthesis, antioxidant agents' regeneration, and amino acid balance. In this scenario, mitochondria can be responsible for chemoresistance fueling 1C metabolism, then enhancing OXPHOS. Lucas *et al.*[37] observed that the activity of SHMT2, a mitochondrial enzyme involved in serine and tetrahydrofolate metabolism, ensures complex I assembly and function, thus increasing the electron transport chain activity and developing resistance.

Resistance to apoptosis is described as one hallmark of cancer, and its contribution to resistant cancer cells is remarkably relevant[38]. Indeed, the correct functioning of mitochondria is fundamental for cellular health. It also has an essential role in programmed cell death, and its morphology is critically important for apoptosis commitment[39]. Changes in the permeability of the inner mitochondrial membrane lead to the loss of the mitochondrial membrane potential allowing the release of pro-apoptotic proteins, such as B-cell lymphoma 2 (Bcl-2) family proteins (Bcl-2, Bcl-xl, Bcl-W, MCL-1, A1, and Bcl-B)[40,41]. Xu *et al.*[42] showed that overexpression of Bcl-2 blocked apoptosis in human ovarian cancer cells. In addition, other pro-apoptotic proteins, such as apoptosis-inducing factor (AIF) and endonuclease G (Endo G), are released from a late event in apoptosis, which occurs once the cells are committed to dying[40]. AIF and Endo G act in a caspase-independent manner to execute cell death[43]. Alvero *et al.*[44] were shown that Endo G mediates caspase-independent cell death in response to chemotherapeutic agents in an ovarian cancer model. One of the aims of chemotherapy is to induce apoptosis by targeting these pro-apoptotic proteins, mainly the Bcl-2 family, creating mitochondrial outer membrane permeabilization, which results in the irreversible release of cytochrome c, caspase activation and then apoptosis[45]. For instance, docetaxel, a common chemotherapeutic agent whose primary mechanism of action is inhibition of microtubule disassembly, can also bind to Bcl-2, inducing apoptosis[46]. Furthermore, a cutting-edge contribution of mitochondria in cancer drug resistance is the exchange of this organelle mediated by tunneling nanotubes, a transient cytoplasmic connection between non-adjacent cells[47]. Several studies have described mitochondrial transfer from important stromal cells of the tumor microenvironment, such as endothelial cells and mesenchymal stem cells, to cancer cells in many types of cancer, such as breast cancer[48], acute myeloid leukemia[49] and glioblastoma[50]. This mechanism promotes tumor survival and chemoresistance by improving OXPHOS or increasing anti-apoptotic proteins.

Mitochondria-targeted drugs for cancer - current status

Since mitochondria are a central organelle for cell survival, including cancer cells, several researchers have dedicated time and effort in recent years to develop new anticancer drugs and strategies that could target mitochondria. This group of compounds, proposed to impair mitochondria, are designated as mitochondria-targeted drugs (MTDs). Their mechanism of action can range from targeting tricarboxylic (TCA) cycle enzymes to electron transport chain (ETC) complexes and Bcl-2 anti-apoptotic family proteins[51]. Some MTDs studied comprise Mito-carboxy proxyl (Mito-CP), Mito-metformin, and Mito-methyl coumarin.

Mito-CP, a lipophilic cationic nitroxide conjugated to an alkyl triphenylphosphonium cation (TPP), showed to accumulate in mitochondria, combined with 2-deoxyglucose (2-DG), a glycolysis inhibitor, in hepatocellular carcinoma and breast cancer cells, inducing apoptosis via caspase 3/7 activation and a significant decrease in intracellular ATP. Notwithstanding, Mito-CP did not affect primary hepatocytes cells or non-tumoral fibrocystic breast cell line (MCF10A), highlighting a possible selectivity of this compound[52,53]. Concerning colorectal cancer, Boyle *et al.*[54] demonstrated for the first time that MTDs induce mitophagy in cancer cells. In the study, Mito-CP and Mito-Metformin, a TPP-conjugated derivative of a common type 2 diabetes drug metformin which acts inhibiting complex I of mitochondrial respiratory chain, released Unc-51-like autophagy-activating kinase 1 (ULK1) from mTOR-mediated inhibition, affecting mitochondrial morphology, and decreasing mitochondrial membrane potential, which are

indicators of mitophagy. Another MTDs is Mito-methyl coumarin, a compound synthesized using the coumarin backbone, which exhibited anticancer proprieties in Hela cells by increasing ROS generation, reducing mitochondrial mass and membrane potential, and inducing programmed cell death[55].

Regarding cancer chemotherapy resistance, Chan *et al.*[56] showed that drug molecules carried by multifunctional nanodiamonds could be effectively delivered to mitochondria and induce remarkable cytotoxicity and cell death in doxorubicin-resistant MCF-7 breast cancer cells. In addition, Li *et al.*[57] synthesized mitochondria-targeted polydopamine nanoparticles loaded with doxorubicin and demonstrated that this system could potentially overcome drug resistance with a short-term treatment plan toward breast cancer cell lines (MDA-MB-231).

Focusing on cancer stem cells (CSCs), MTDs are considerably more effective in triggering apoptosis of CSCs, when compared to other agents, via mitochondrial dysfunction by regulating Bcl-2 family proteins, and ROS production[58]. Thus, specific mitochondrial-targeted compounds can induce cell death in chemoresistant CSCs. For example, Hirsch *et al.*[59] showed that metformin, an inhibitor of the complex I mitochondrial respiratory chain, selectively kills CSCs in breast cancer cell lines. Alvero *et al.*[60] demonstrated that the novel isoflavone derivative NV-128 significantly decreased mitochondrial function and induced cell death in ovarian CSCs.

Although only one MTD has been approved for cancer treatment (BH3 mimetic Venetoclax) so far, other drugs have shown beneficial and promising effects in phase I/Ib and phase I clinical trials, such as MitoTam (EudraCT 2017-004441-25)[61] and IACS-01075917 (NCT03291938)[62], respectively. Given the importance of mitochondria for cancer cells, especially cancer stem cells, further research on agents that target mitochondria and combining these drugs with other chemotherapeutics are encouraged to increase the efficiency of anticancer therapies.

CANCER STEM CELLS AND MITOCHONDRIA: A STRONG PAIR IN DRUG RESISTANCE IN CRC

As already described, mitochondria are involved in many metabolic pathways, and alterations in this metabolism have been found in CSCs[24]. Huang *et al.*[63] discovered that suppressing mitochondrial ROS production drives glioma stem-like cell progression and facilitates radiotherapeutic resistance. In addition, Ren *et al.*[64]. observed alterations in mitochondrial function, such as the low amount of mtDNA and oxygen/glucose consumption and low intracellular ROS and ATP concentrations in thyroid cancer stem cells, which contribute to radioresistance. Kuntz *et al.*[65] indicate that alterations in essential mitochondrial functions, such as oxidative phosphorylation, contribute to therapy-resistant chronic myeloid leukemia stem cells.

The damage of mtDNA, or even its loss, causes a reduction in the proliferation rate of CSCs[27]. Huang *et al.*[66] show that mtDNA deficiency may induce ovarian cancer stem cell-like properties and reveal the downregulation of mitochondria-related genes and upregulation of genes related to cell proliferation, anti-apoptosis, and drug resistance. Although these data indicate mitochondrial dysfunction and OXPHOS reduction, it has been reported the coexistence of a CSCs subpopulation that produces ATP via an anaerobic glycolytic pathway and feeds the OXPHOS of the OXPHOS-addicted subpopulation. This metabolic shift, known as the Warburg effect, usually occurs in regions of tumor hypoxia[67]. Genetic and epigenetic alterations were found in the mtDNA of cisplatin-resistant oral squamous cancer cells. Further study of these changes may help to elucidate their role in chemoresistance[68].

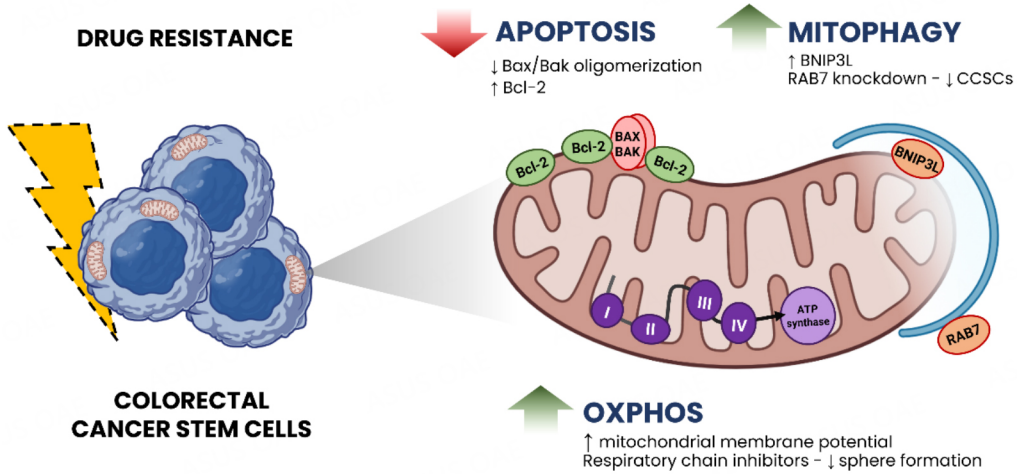

Figure 1. Mitochondria involvement in colorectal cancer stem cells and drug resistance. (1) Reduced apoptosis due to lower Bax/Bak oligomerization and higher Bcl-2 expression. (2) Enhanced mitophagy with the participation of RAB7 and higher BNIP3L expression. (3) Addiction to OXPHOS with high mitochondrial membrane potential. The vectors used in the figure are from BioRender©. BNIP3L: BCL2 Interacting Protein 3 Like; CCSCs: colorectal cancer stem cells; OXPHOS: oxidative phosphorylation; RAB7: Ras-related protein 7.

Concerning apoptosis, CSCs survival depends on the dysregulation of apoptosis pathways and the overexpression of anti-apoptotic proteins[69]. The anti-apoptotic proteins include the cellular FLICE-inhibitory protein (c-FLIP), the Bcl-2 family of proteins, and the inhibitor of apoptosis proteins (IAPs). Piggott *et al.*[70] showed that the overexpression of c-FLIP plays a crucial role in resistance to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, an anticancer agent, in breast cancer stem cells. Guo *et al.*[71] indicated that survivin/BRIC5, a member of the IAPs family, is promising to be an excellent candidate for recombinant anti-cancer protein by promoting apoptosis of cancer cells and their stem cells through sensitizing cells to chemotherapeutic drugs.

Furthermore, CSCs and mitochondrial dynamics are valuable partners for overcoming drug resistance[72]. Brain CSCs derived from tumor xenografts and primary tumor samples showed higher fragmented mitochondria and activation phosphorylation of dynamin-related protein 1 (DRP1) compared to non-CSCs[73]. Notwithstanding, in a breast cancer model, mitochondrial fusion promoted by upregulation of MCL-1 showed to maintain CSCs properties in cooperation with MYC, facilitating mitochondrial respiration, thus inducing chemotherapy resistance[74].

Apoptotic proteins expression

In CRC, there is an intimate relationship between CSCs and mitochondria in drug resistance scope [Figure 1]. Regarding the apoptosis mitochondrial pathway, recent data ensure that anti-apoptotic protein overexpression is vital for CCSCs chemoresistance. Purushothaman *et al.*[75] showed that untreated $CD133^+$ CCSCs (HCT116) expressed lower Bax/Bak oligomerization and higher Bcl-2 in comparison to Ruthenium(II) complex 1-treated group. In agreement with these findings, Colak *et al.*[76] observed that CCSCs have decreased mitochondrial priming, accumulating anti-apoptotic proteins and inducing chemoresistance instead of differentiated CRC cells. In contrast, CCSCs incubated with WEHI-539, a specific inhibitor of Bcl-xl, were more sensitive to oxaliplatin, indicating that the mitochondrial apoptosis pathway is a great potential target for drug-resistant CCSCs. Ramesh *et al.*[77] demonstrated that treatment with a BCL-XL inhibitor (A-1155463) increased the percentage of cells with active caspase-3 in Wnt-high CCSCs, indicating cell death, while BCL-2 (ABT-199) and MCL-1 (AZD5991) inhibitors did not promote this effect. These data suggest that CCSCs may depend only on BCL-XL and not on BCL-2 and MCL-1,

indicating the therapeutic potential of inhibiting BCL-XL activity.

Mitophagy rates

Mitophagy has also been assessed in CCSCs as it is another important mechanism for therapy resistance in cancer. Takeda *et al.*[78] demonstrated that mitophagy was enhanced among CCSCs compared to non-CCSCs, and the knockdown of RAS-Associated proteins (RAB5 and RAB7) diminished the $CD44v9^+$/$CD133^+$ CCSCs population. Yan *et al.*[79] showed that $CD44^+$/$CD133^+$ CCSCs (HCT8 cell lineage) were more resistant to doxorubicin treatment and expressed higher levels of BNIP3L, a mitophagy-related protein concerning parental cells. Instead, when CCSCs were treated with siRNA for BNIP3L, they became more sensitive to doxorubicin, suggesting that mitophagy is a major strategy of CCSCs to escape from death.

Oxidative phosphorylation addiction

Regarding metabolism, Denise *et al.*[80] showed that 5-FU resistant colorectal HT29 cancer cells are addicted to OXPHOS while expressing more CD133 and forming more spheres, a 3D cell culture, *in vitro*. The coadministration of 5-FU and respiratory chain complex inhibitors led to a shrinkage of spheres. Corroborating to these findings, Song *et al.*[81] demonstrated that $CD133^+$/$CD44^+$/$Lgr5^+$ CCSCs were highly dependent on OXPHOS and presented high mitochondrial membrane potential. Besides, it was observed that the incubation with 5-FU did not alter ATP levels of $CD133^+$ CCSCs compared to non-CCSCs. Combinate treatment of 5-FU and antimycin A, an inhibitor of complex III from the respiratory chain, promoted cell death along with high expression of cleaved caspase 3. Thus, the addiction to OXPHOS appears to support CCSCs phenotype.

CONCLUSION

Colorectal cancer remains a disease of great concern as therapy resistance remains a remarkable challenge. In this scenario, cancer stem cells are well-known contributors, and their mitochondria exhibited a valuable role in providing it. A major mechanism for chemotherapeutic avoidance in CRC is an enhancement in anti-apoptotic protein expression among CSCs, a higher mitophagy rate and a reliance on OXPHOS.

In this concern, studies involving approaches such as patient-derived xenografts and organoids should be conducted to expand the understanding of the correlation of CCSCs and their mitochondria, regarding energetic metabolism, apoptosis pathway, especially mitophagy, providing a better picture of the heterogeneity intrinsic to CRC and its chemoresistance. The literature also lacks studies about mitochondria morphology in CCSCs in drug resistance context, which is a potential topic for new remarks. Moreover, new chemotherapy strategies targeting mitochondria must be studied and developed aiming at the elimination of CCSCs and their great contribution to CRC chemotherapy resistance.

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Critical revision: Thole AA, Mencalha AL

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Commentary

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Fungal mycobiome-mediated immune response: a non-negligible promoter in pancreatic oncogenesis and chemoresistance

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers in humans due to late diagnosis and poor response to treatments. The tumor microenvironment (TME) of PDAC is characterized by a distinctive, suppressive immune profile, which inhibits the protective functions of anti-tumor immunity and thereby contributes to PDAC progression. Recently, the study of Alam *et al.* discovered for the first time that the intratumoral fungal mycobiome could contribute to the recruitment and activation of type 2 immune cells in the TME of PDAC via enhancing the secretion of a chemoattractant, interleukin (IL-) 33. In this article, we reviewed the important findings of this study. Together with our findings, we synthetically discussed the role of the fungal mycobiome in orchestrating the immune response and thereby modulating tumor progression.

Keywords: Mycobiome, pancreatic cancer, chemoresistance, interleukin 33, type 2 immune cells

MAIN TEXT

The recent development of various –omics approaches has greatly expanded our knowledge of the diverse fungal species colonizing different body sites, which constitute an important component of the human microbiome, termed the "mycobiome"[1,2]. Traditionally, members of the human mycobiome, such as the well-known *Candida* spp., are known as opportunistic pathogens which reside in most healthy individuals and cause local or systemic infectious diseases only under certain circumstances. However, a growing body of evidence has suggested that the mycobiome plays a critical role in the onset and progression of cancers[3]. A noteworthy example is the implication of the mycobiome in the carcinogenesis of pancreatic ductal adenocarcinoma (PDAC).

PDAC is a highly aggressive malignancy with a 5-year overall survival (OS) rate of around 10%, ranking the fourth leading cause of cancer-related deaths in the Western world[4]. The dismal prognosis mainly results from a lack of specific symptoms for early diagnosis, the early metastatic spread and the poor response to available treatments[5]. Previously, a seminal study discovered that PDAC harbors a distinct mycobiome profile as compared to that of the normal pancreas, while fungal ablation with antifungal treatments showed protective effects against oncogenic progression[6]. These results suggested the potential of the mycobiome in PDAC as a new target for the development of novel biomarkers and therapeutic strategies[7].

The tumor microenvironment (TME) of PDAC is characterized by a distinctive immune profile dominated by immune-suppressive cells including T_H2 cells and innate lymphoid cells 2 (ILC2), which can inhibit the functions of anti-tumor T cell immunity and thereby contribute to PDAC progression[8]. In addition, these infiltrated T_H2 cells have also been found to fuel PDAC progression in the early stage of tumorigenesis via the secreted type 2 pro-tumorigenic cytokines, such as interleukin (IL-) 4 and IL-13[9].

Most recently, Alam and collaborators[10] revealed for the first time that the intratumoral mycobiome could enhance the secretion of the chemoattracting cytokine IL-33 from cancer cells, which subsequently recruited and activated T_H2 and ILC2 cells in the TME of PDAC, thus promoting pancreatic oncogenesis. This study was the first to show that TME of PDAC has an increased infiltration of T_H2 and ILC2 cells as compared to the normal pancreas both in a PDAC mouse model and in human PDAC samples[10]. In order to determine the chemotactic factors secreted by cancer cells that may recruit and activate these immune cells, the authors also conducted a transcriptomic analysis of multiple PDAC cell lines and identified a 30-fold upregulation of IL-33, which was mediated by oncogenic *Kras*^{G12D} signaling. Immunohistochemistry staining showed that IL-33 expression was indeed relatively high in human PDAC tissues, while it was undetectable or below 25% nuclear staining in exocrine cells in normal pancreas specimens in the Human Protein Atlas database (https://www.proteinatlas.org, Figure 1A). IL-33 is known as a potent activator of T_H2 and ILC2 cells[11]. In order to elucidate the requirement of IL-33 expression by cancer cells to recruit type 2 immunocytes, the authors depleted IL-33 in cancer cells by lentivirus transduction of small hairpin RNA in a syngeneic orthotopic model of PDAC. The IL-33 depletion reduced both T_H2 and ILC2 infiltration in the TME and functionally inactivated the resident ILC2 cells that were already present within the TME. The decreased IL-33 expression in cancer cells also resulted in reduced tumor burden and increased survival. Taken together, these results indicated that cancer-cell-derived IL-33 recruits and activates type 2 immune cells into the TME of PDAC.

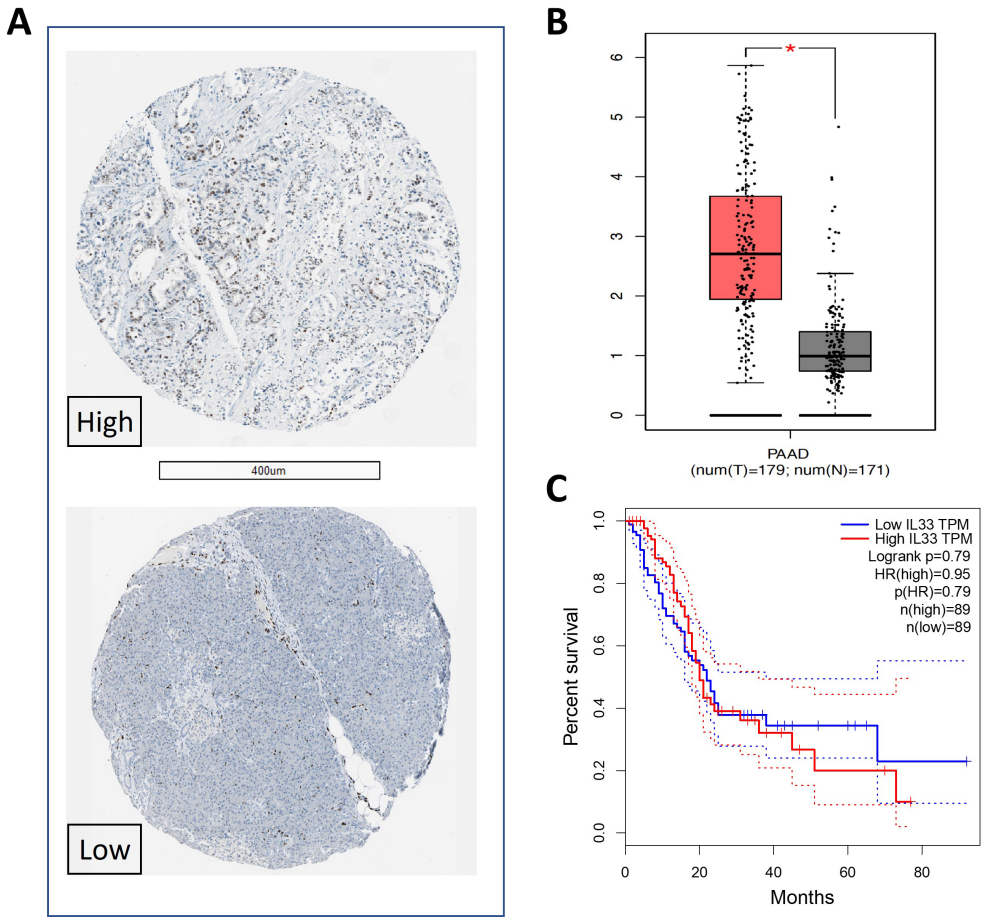

Figure 1. IL-33 expression and outcome in PDAC. (A) Representative images showing differential expression (high *vs.* low) of IL-33 in cores of tissue microarrays including specimens from two PDAC patients[24]. The immunohistochemical staining was performed using the IL-33 antibody AF3626 (R&D systems), as reported by Alam *et al*[10]; (B) the mRNA expression of IL-33 in PDAC was evaluated using the web-based genomics analysis and visualization platform GEPIA, analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from the TCGA and the GTEx projects, including 179 PDAC and 117 normal pancreatic tissues; (C) the levels of IL-33 mRNA expression did not correlate with overall survival in the TCGA-PAAD database.

Other experiments focused on the role of the mycobiome in PDAC on IL-33 secretion as well as in PDAC tumorigenesis[10]. Using both 18S internal transcribed spacer (ITS) sequencing and fluorescence *in situ* hybridization, a higher load of fungi was found in the PDAC specimens as compared to the normal pancreas, with *Malassezia* being the most abundant genus, in accordance with previous findings[6]. In addition, in both studies, the fungal depletion or repopulation was shown to retard or accelerate PDAC tumor growth, respectively. However, various mechanisms may be responsible. *M. globosa* was shown to promote tumor progress[6] via mannose-binding lectin that can recognize fungal pathogens and the subsequent activation of C3 complement cascade which belongs to innate immunity[12]. Interestingly, *M. globosa* was also shown to be involved in regulating the adaptive immune response to promote tumor growth by facilitating the extracellular expression of IL-33 and consequently enhancing the infiltration of T_H2 and ILC2 cells[10].

However, IL-33 is a member of the IL-1 cytokine family[13] and it is well known for its dichotomous functions, acting both as a traditional extracellular cytokine and as a nuclear transcription factor[14]. Unlike the traditional inducible cytokines, IL-33 is constitutively expressed by several cells including human

endothelial and epithelial cells. The full-length IL-33 can translocate to the nucleus upon synthesis and be stored there[15,16]. This nuclear IL-33 might function as a transcriptional repressor to decrease inflammation[17]. Once released or secreted, the extracellular IL-33 can be cleaved to its more active form and induce the type 2 immune response[11,18]. Interestingly, there are different opinions on the function of type 2 immune response in intestinal immunity and in the development of pancreatitis and PDAC. Some studies showed that IL-33 deficient mice were highly susceptible to colitis, colorectal cancer and pancreatitis[19,20], which suggest a protective function of IL-33. However, other studies showed increased IL-33 levels in biopsies obtained from patients with active inflammatory bowel disease[21], while elevated serum IL-33 was found in patients with severe acute pancreatitis[22]. Opposite findings described that the IL-33-induced ILC2 infiltration in PDAC cells correlated positively with long-time survival in patients[23]. In The Cancer Genome Atlas (TCGA) database, the mRNA expression of IL-33 is significantly higher in PDAC tissues compared to normal pancreatic tissues but was not associated with a significantly different OS [Figure 1B and 1C].

However, in many human cancers including PDAC, it was found that the fragile X mental retardation protein (FMRP) repressed immune attack by up-regulating IL-33 together with tumor-secreted protein S and extracellular vesicles (EVs), which promote M2-like tumor-associated macrophages, while down-regulating the chemoattractant C-C motif chemokine ligand 7[25]. Of note, FMRP mRNA and protein expression levels were not associated with clinical outcomes in several cohorts of cancer patients, but a gene signature reflecting FMRP's cancer regulatory activity (with 156 genes, including IL-33) was prognostic for reduced OS across multiple human cancers. These discrepancies underline the importance of understanding the role of IL-33, its induced type 2 immune response and the network of genes and cells in the TME that contribute to the capability of PDAC to evade immune destruction and resist chemotherapy. Elucidation of the processes is essential before designing novel treatment strategies.

Since the mycobiome is living in symbiosis with bacteria as commensals in the human body, it is very likely that the mycobiome exerts an important influence on the microbiome. The involvement of microbiome in the development of PDAC and its chemoresistance has been convincingly demonstrated by several studies[26-29]. Bacterial taxa, Proteobacteria (*Pseudoxanthomonas*) and Actinobacteria (*Saccharopolyspora* and *Streptomyces*), are positively correlated to the short-term survival of PDAC patients[29]. The 18S ITS sequencing can only detect the presence of fungal mycobiome but not the presence of microbiome. It is therefore unclear whether microbiome could function similarly to the mycobiome, inducing the secretion of extracellular IL-33 and thus activating the type 2 immune response. The factors which can trigger the release or secretion of extracellular IL-33 are not fully identified. Besides fungi or fungal components[10], it has been shown that cellular injury or death is one of the mechanisms by which IL-33 reaches the extracellular environment[30]. Hence IL-33 will also act as an alarm when there is a breach in the primary defenses of intestinal epithelium against pathogens and other threats[18]. Other factors, such as extracellular ATP concentrations, mechanical stress or oxidative stress, can also enhance the secretion of IL-33. Extracellular ATP concentrations are regulated by ectonucleotidases CD39 and CD73, which are known to play a role in immune function as well[31,32]. Multiple bacterial species, such as *Klebsiella pneumoniae* (for autoimmune pancreatitis)[33], *Helicobacter pylori* (for gastric ulcers)[34], *Staphylococcus aureus* (for lung infection)[35], have been shown to enhance the secretion of IL-33, indicating the involvement of tumoral microbiome, possibly together with mycobiome, in orchestrating the innate and adaptive immunity and modulate tumor progression.

Furthermore, mycobiome may also play a role in chemoresistance. Aykut *et al.*[6] have shown that fungal ablation via antifungal treatment enhanced the efficacy of gemcitabine-based chemotherapy in PDAC-

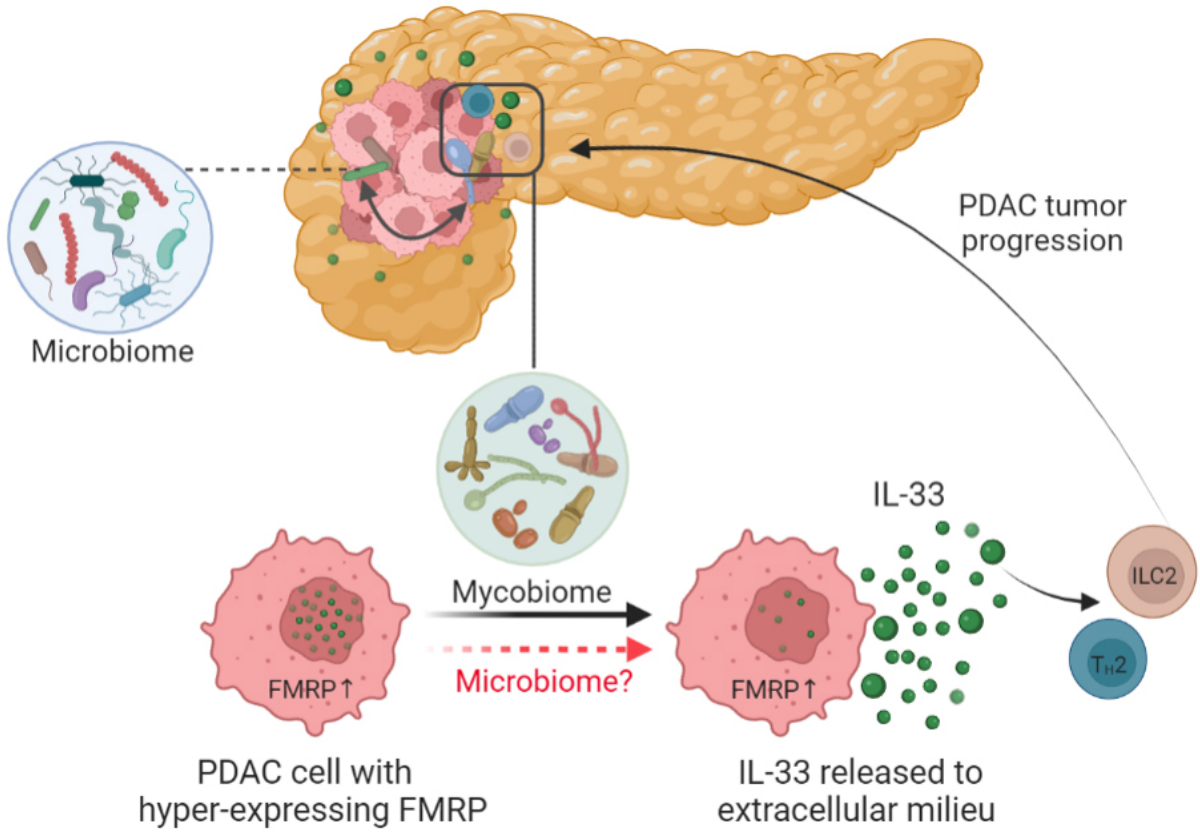

Figure 2. Intratumoral mycobiome can facilitate the extracellular secretion of interleukin (IL-) 33 from the nuclear of pancreatic ductal adenocarcinoma (PDAC) cells, which recruit the pro-tumorigenic immune cells such as T_H2 and ILC2 cells to the tumor microenvironment and thus accelerate PDAC tumor progression. Notably, bacteria species have been shown to be able to enhance the secretion of IL-33, and possibly together with mycobiome, intratumoral microbiome may also exert a role in orchestrating the host immune response and modulating tumor progression. FMRP, fragile X mental retardation protein. (Created with BioRender.com).

bearing mice. In humans, it was suggested that the gut mycobiome might modulate the response to preoperative chemotherapy (gemcitabine-cisplatin) in patients with bladder cancer[36]. Compared to the non-responders, the responders had a distinct mycobiome featured by a higher diversity and lower abundance of *Agaricomycetes* and *Sacchaaromycetes*. However, the mechanisms underlying mycobiome-induced chemoresistance are still unclear. There are several hypotheses: (1) mycobiome may confer chemoresistance through metabolism and enzymatic degradation of chemotherapeutic drugs, similar to the previously reported intratumoral bacteria-mediated chemoresistance[27]; (2) studies on breast cancer have identified IL-33 as a key driver of chemoresistance of tumor cells. IL-33 overexpression transformed tumor cells into polyploidal giant cancer cells that are highly resistant to chemotherapy due to their dormancy or abnormal cell cycle[37,38]. Thus, the fungal mycobiome may also elicit chemoresistance of cancer cells via the enhanced secretion of IL-33, as shown in the current evaluated study of Alam *et al.*[10].

In summary, recent data revealed that the intratumoral fungal mycobiome can contribute to PDAC pathogenesis by stimulating the extracellular secretion of IL-33 from cancer cells, thus driving the recruitment and activation of T_H2 and ILC2 cells in TME of PDAC and promoting tumor progression [Figure 2]. Recent studies suggest that additional mechanisms, including modulation of FMRP and interaction with other fungi and bacteria, play a pivotal role in the impact of IL-33 as "friend or foe" in PDAC [Figure 2]. These findings may provide new insights for the development of novel therapeutic

strategies for overcoming PDAC chemoresistance by targeting the intratumoral mycobiome and correlated factors. Nevertheless, research on fungal mycobiome in PDAC is still at the infant stage. More studies are needed to illustrate how fungal mycobiome and its interaction with intratumoral bacteria can influence the oncogenesis and chemoresistance of PDAC. Furthermore, clinical studies should also be conducted to understand the prevalence of fungal infection in PDACs, the heterogeneity of IL-33 expression and the tumor-stage associated IL-33 expression, which may assist in the discovery of novel biomarkers for monitoring disease progression.

DECLARATIONS

Authors' contributions

Writing - original draft preparation: Jiang Y, Donati V
Writing - review and editing: Peters GJ, Giovannetti E, Deng DM
All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data supporting the findings of Figure 1 are available from the authors upon request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Remodeling the tumor microenvironment to overcome treatment resistance in HPV-negative head and neck cancer

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Abstract

Despite intensive efforts and refined techniques, overall survival in HPV-negative head and neck cancer remains poor. Robust immune priming is required to elicit a strong and durable antitumor immune response in immunologically cold and excluded tumors like HPV-negative head and neck cancer. This review highlights how the tumor microenvironment could be affected by different immune and stromal cell types, weighs the need to integrate metabolic regulation of the tumor microenvironment into cancer treatment strategies and summarizes the emerging clinical applicability of personalized immunotherapeutic strategies in HPV-negative head and neck cancer.

Keywords: Tumor microenvironments, head and neck cancer, SBRT, immunotherapy, metabolic reprogramming, radiotherapy, HPV-negative

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) develop in close proximity to the anatomical structures responsible for breathing, speaking, chewing, and swallowing. These structures include the pharynx, larynx and oral cavity. HNSCC are common worldwide, with approximately 880,000 new cases and 445,000 deaths (excluding salivary glands) in 2020[1], and are expected to increase by 42% by 2040[2]. Up to 60% of patients

with HNSCC present with locoregionally advanced disease[3] and usually receive multimodal treatment combining surgery, radiation therapy (RT), and chemotherapy. Even following vigorous treatment, recurrences in the same area or elsewhere have a poor prognosis[3]. As a result, there is a great deal of morbidity associated with the treatment for HNSCC, whether it is intended to slow the disease's course or to cure it entirely. More importantly, HNSCC survivors experience one of the highest rates of suicide likely influenced by the accompanying psychosocial distress and reduced quality of life[4]. Multidisciplinarity is a cornerstone of HNSCC management[5] that incorporates diagnostic, therapeutic, prognostic, and patient care collaborative reasoning in the decision-making process. Consensus guidelines are a valuable resource in challenging situations and ensure the quality of tumor board decisions[6]. Finally, emergent strategies like the impact of the microbiome in HNSCC can be readily incorporated into multidisciplinary treatment planning[7].

The current treatment strategy is based on tumor location and disease stage, making tumor burden a strong determinant for the treatment decision and outcome, though HPV-positive tumors are associated with a better prognosis[8]. Despite recent technological advances aimed at reducing treatment-related toxicities[9-11], local control and overall survival rates in locally advanced disease are rather low, ranging from 40% to 50% at 5 years, especially in HPV-negative cancer[12]. Recent developments in immunotherapy have revealed the importance of the tumor microenvironment (TME) in participating in effective immune responses. In recurrent/metastatic HNSCC (R/M-HNSCC), immunotherapy is now a mainstay of treatment, with objective response rates of 13%-17% in an unselected R/M-HNSCC population and 2-year overall survival rates of 17%-27%[13-15], but immunotherapy has failed in the curative setting[12]. Equally relevant, genetic and epigenetic events define treatment response. In that regard, it has been shown that HPV-positive tumors with excellent antitumor response harbor a defective DNA repair[16]. However, not all HPV-positive tumors hold a better prognosis[17]. It is well known that outcomes are poorer for HPV-negative cancers, including those in early stages. In summary, even within histology, tumors may have markedly differing sensitivities.

Hypoxia, which is a hallmark of HNSCC, is a significant impediment to the effectiveness of radiation therapy (RT). Hypoxia, which reduces the induction of DNA double-strand breaks in low oxygen conditions[18] and induces tumor cells to enter a state of quiescence[19], is the cause of radiation resistance. This is essentially the result of aberrant tumor vasculature as well as the high oxygen consumption of a tumor cell population that is expanding at a rapid rate. Both the failure of the treatment and the development of tumor resistance have been connected to hypoxia-induced changes in cellular redox as well as the utilization of alternative metabolic pathways in the TME. Future efforts should focus on differentiating between patients with severely and/or non-correctable hypoxic tumors and those with milder hypoxic characteristics.

Neck nodal disease is common and a well-established adverse prognostic factor in HNSCC[20]. Occult metastases in the cervical lymph nodes may be present in patients even if their primary tumors were not particularly large. This has traditionally resulted in the inclusion of elective treatment of the neck as part of the curative therapeutic strategy, being regarded as a factor contributing to survival[21]. However, de-escalation strategies suggest that treatment intensity can be modulated. For example, unilateral neck treatment for lateralized oral cavity and oropharyngeal tumors[22] or reduction of RT dose and/or volume to the elective neck in HNSCC[23,24]. In the 30 ROC trial, treatment volume and radiation dose were reduced by 60% (i.e., to 30 Gy) in HPV-positive oropharyngeal tumors obtaining excellent results in non-hypoxic tumors[16]. Furthermore, recent preclinical models suggest that elective nodal irradiation reduces the efficacy of combined stereotactic radiotherapy and immunotherapy[25]. Cumulative evidence suggests that functional

lymphatics promote an effective immune response. In contrast, lymphatic remodeling promotes tumor proliferation, indicating that tumor lymphangiogenesis is a causal factor in tumor immune surveillance by the host[26]. Preclinical studies have shown that lymph node tumor cells have the ability to reach other organs via high endothelial venules[27,28], which is an intriguing fact to take into consideration. As a result, the development of novel treatments is made possible thanks to a deeper comprehension of the dynamic relationship that exists between lymphatics, tumor cells, and the TME.

This review explores how immune and stromal cells in the TME influence immunosuppression and proposes targeting strategies to promote immunomodulation, weighs the integration of metabolic regulation of the TME into treatment strategies, and summarizes the emerging clinical applicability of personalized immune therapies in HPV-negative HNSCC.

KNOWLEDGE GAPS IN THE CURRENT THERAPEUTIC LANDSCAPE IN HPV-NEGATIVE HNSCC

A high tumor mutational burden is characteristic of HNSCC, and this has been identified as a critical factor in immune response in other malignancies. Furthermore, HNSCC tends to exhibit tumor-infiltrating immune cells, which are a recognized prognostic factor in a subset of HNSCC subtypes. However, the impact of immunotherapy on HNSCC has been rather low, with HNSCC falling into certain subtypes and that have a high level of resistance[29,30]. Reevaluating the treatment for these tumors, which are not immunogenic, is one way to improve the standard of care for these cancers.

Prognosis

The stage and HPV status are recognized as the major determinants of HNSCC prognosis. However, it is becoming clear that not all HPV-positive HNSCC share the same good prognosis[17] and that not all HPV-negative HNSCC are the same[31].

Anatomical location, patterns of lymphatic drainage and patient preference, in combination with technical/technological requirements/skills and foreseen toxicities delineate curative treatment strategies in HNSCC. Nevertheless, hypoxia and the TME strongly affect the efficacy of immunotherapy[32]. An understanding of the relative variations in the different anatomical sites of HNSCC might assist in the planning of new and more effective treatment strategies. A recent evaluation of the TCGA dataset concludes that HPV-negative sites are molecularly different, especially between tumors of the oral cavity and larynx[31]. In this study, larynx cancer had a higher mutational burden, and was enriched for neuronal and glycosylation pathways, with a greater abundance of B cells and endothelial cells; while oral cavity cancer was enriched in extracellular matrix (ECM) pathways, with a greater abundance of monocytes and greater methylation of Hox genes; oropharyngeal cancer was the most hypoxic, and oral tongue cancer had a higher abundance of dendritic cells (DCs).

The immune system's decreased recognition of the tumor or inhibition of its response may explain why some HNSCC respond to immunotherapy, but many others behave as resistant and are regarded as poor immunogens (see excellent review in ref.[33]).

Landscape of the immunosuppressive TME in HPV-negative HNSCC

The TME is heterogeneous and can be envisioned as a core of specialized microenvironments disposed as intersecting paths that can reprogram cancer biology and serve as potential targets of cancer therapy [Figure 1].

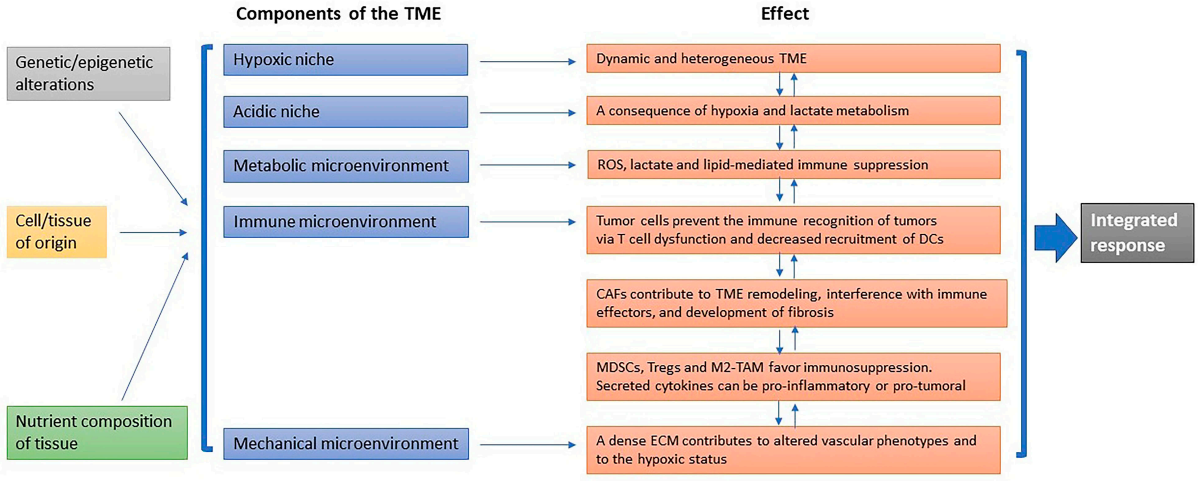

Figure 1. Dynamic composition of the TME. Specialized microenvironments that can be influenced by the genetic/epigenetic background, the tissue of origin, and the components of the tissue, and promote specific effects that are dynamically interconnected. The final outcome results from an integrated response.

Spatial architecture of the tumor versus immune evasion

A global hypoxic state is facilitated by a dense ECM, which limits immune cell infiltration and prevents their antitumor effect. Increased angiogenesis, the recruitment of additional immunosuppressive cells, tumor progression and enhanced metastatic ability are all factors that contribute to a global hypoxic state. Tissue microarrays of human HNSCC confirmed the vascular heterogeneity observed in patient-derived xenograft (PDX) models of HNSCC. These models showed three distinct vascular phenotypes, including a tumor vessel (TV) phenotype in which the majority of the blood vessels were distributed throughout the tumor; a stromal vessel (SV) phenotype in which the majority of the vessels were restricted to the infiltrating stroma next to the tumor cells; and a mixed vessel phenotype. In HPV-negative HNSCC, a heterogeneous distribution of vascular phenotypes was seen, with 60% of the tumors belonging to the SV phenotype, 25% of the tumors belonging to the mixed vessel phenotype, and 15% of the tumors belonging to the TV phenotype. A better response to antivascular agents was seen in the TV phenotype, but not in the SV phenotype[34]. Understanding the physical barriers involved in HNSCC opens up the possibility for localized drug delivery, which could limit the systemic effects of different immunotherapies.

Tumor cells versus immune recognition of tumors

Conditions that prevent the proper priming, activation and infiltration of T cells are known to compromise the immune response. Thus, the T cells that are present in poorly immunogenic tumors are dysfunctional, exhausted or excluded from the TME. T cell exhaustion (TEX) is considered a major limitation to the long-term efficacy of immunotherapy. The complex immunosuppressive network in the TME facilitates the majority of $CD8^+$ T cells evolving into the exhausted T cell subtype[35]. TEX is not a static process with only one conceivable end; rather, it comprises a large variety of transitional phases. Instead, the pool of exhausted $CD8^+$ T cells that are found in the TME should be considered as a population of $CD8^+$ T cell subsets that have varying degrees of TEX, and each has its own unique set of functional capabilities [36]. In this regard, a pan-cancer analysis of the heterogeneous TEX subset landscape using a five-stage trajectory as measured using TEX-specific hierarchical developmental signaling pathway signatures reveals the interdependencies of the TEX subgroups in various types of tumors, as well as among the same types of tumors. TEX patterns in HNSCC TCGA molecular subgroups revealed that the highly immunogenic progenitor TEX (TEX^{prog}) subset was particularly enriched in the HNSCC basal type, while the three

remaining molecular types (atypical, mesenchymal and classical) demonstrated intermediate distribution of transcriptional factors T cell factor 1 (TCF1, marks a downstream population of stem-like precursors of $CD8^+$ T cells characterized by high self-renewal capacity, proliferation, and polyfunctionality[37]), T-box expressed in T cells (T-bet; involved in effector, memory and exhausted $CD8^+$ T cell differentiation[38]), and thymocyte selection-associated high mobility group box (TOX, essential role in the induction of TEX by mediating transcriptional and epigenetic changes) known to coordinate the dynamics underlying TEX subset transitions[39].

Decreased recruitment of DCs, required for tumor antigen presentation, can also contribute to immune escape by preventing adequate T cell priming and trafficking into the TME. Both classical DC (cDC1, cDC2) and plasmacytoid DC (pDC) are distinct subsets of the DC compartment. Batf3-driven $CD8\alpha^+$/$CD103^+$ cDC1 are thought to be the major mediators of antigen transport and cross-priming since they are the most common cross-presenting DC subgroup. While cDC2/moDC and pDC have been shown to have stimulatory roles in some contexts, these cells' roles are often more tolerant in the TME [40]. Tissue-specific DCs and tumor-associated macrophages (TAMs) can be generated in the environment of the tumor, inhibiting the anticancer immune response. The relevance of tissue-derived APCs to anticancer immune responses is highlighted by the plasticity of the myeloid compartment in response to the microenvironment. Learning how tissue-specific APCs differ in their function will aid in the discovery of cutting-edge cancer immunotherapies[41].

It is common knowledge that various other cell types are also capable of carrying out the process of exogenous antigen presentation. These cell types are grouped together and referred to as "amateur" APCs[42]. There is accumulating evidence to suggest that the expression of HLA class II antigens on tumor cells has a major impact on immunogenicity[43]. There is a correlation between favorable outcomes and high levels of constitutive HLA class II antigen expression in oropharynx cancers[44]. It is possible that the favorable effect of HLA class II antigen expression is due to the presence of high amounts of interferon gamma (IFNγ) in tumors that have substantial infiltration by T cells.

Immunosuppressive cells in the TME vs .immune effectors

Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) participate very actively in the TME remodeling and interfere with immune cell effectors, rendering them ineffective. High CAF density is correlated with disease stage and poor prognosis in HNSCC[33]. CAF-induced recruitment of regulatory T cells (Tregs) through transforming growth factor beta (TGF-β) and interleukin 6 (IL-6) inhibits the proliferation of $CD8^+$ T cells in HNSCC[45].

CAFs-TME remodeling. CAFs are the most common type of cell to be seen in the stroma of a tumor. These CAFs secrete cytokines, which can either act on many immune cells at the same time or affect the TME in some other way to influence immune cell infiltration. CAFs are responsible for the release of soluble molecules, such as IL-6, which have an effect on T cells, NK cells, DCs, TAMs, and neutrophils. Single-cell RNA sequencing has previously been used to identify three distinct CAF types in HNSCC; these types include myCAF and two undefined CAF subtypes (CAF1 and CAF2). Despite this, the functional significance of these subtypes and their relationship to the immunotherapy response are still unknown[46]. A study that used protein activity patterns, as determined by the VIPER algorithm analysis of a longitudinal single-cell transcriptomics HNSCC dataset[47], indicated that there are five CAF subtypes that are distinct from one another on a molecular level. The HNCAF-0 and HNCAF-3 subtypes were predictive of favorable clinical responses to PD-1 immune checkpoint blockade (ICB), and they were connected to improved $CD8^+$

T cell cytotoxicity (pro-inflammatory CAFs). Tissue-resident memory (Trm) phenotype $CD8^+$ T cells were created by co-culturing HNCAF-0/3 with $CD8^+$ T cells. These cells co-expressed CD94/NK group 2 member A (NKG2A, CD159), an inhibitory receptor that is substantially abundant in tumor-infiltrating Trm^+ $CD8^+$ T cells in HNSCC[48]. As activating NKG2A with its ligand HLA-E reduces cytotoxicity and effector function, this protein has the potential to be used as a new target for immunotherapy[49]. In clinical trials involving HNSCC, the use of a combination of NKG2A inhibition and other checkpoint inhibitors has been shown to have a positive effect on the patients[50]. Additional evidence has been reported in bladder cancer, suggesting that bladder tumors having both high levels of HLA-E and NKG2A-positive $CD8^+$ T cells could benefit the most[49], and in radioresistant tumors that do not respond to combined RT and ICB[51]. High-resolution single-cell sequencing CAF analyses constitute an excellent framework to develop strategies to reprogram CAFs towards the pro-inflammatory phenotype, identify novel combinations with immunotherapy, and the potential to establish HNCAF subtypes as biomarkers of response and resistance in future clinical trials[47].

The proliferation of CAFs is a critical step in the development of fibrosis in the tumor stroma[52]. Multiple studies have pinpointed a critical function for the anti-apoptotic protein Bcl-2-associated athanogene 3 (BAG3) in tumor cell signaling in the TME[53] and in the progression of fibrosis in tumor tissues[54]. BAG3 causes TAM activation and IL-6 production in pancreatic tumor cells. The close link of BAG3 expression with cancer fibrotic phenotypes[55] suggests that anti-BAG3 therapy dramatically down-modulated the expression of a-SMA, an activation hallmark of CAFs, with a marked reduction of collagen buildup. HNSCC has been identified as a fibrotic tumor phenotype that is more likely to respond to anti-BAG3 therapy, as determined by an analysis of three distinct databases including high-throughput RNA sequencing information from PDXs[56].

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) trafficking-metabolism. Monocytic (M-MDSC), granulocytic polymorphonuclear (PMN-MDSC), and early stage (e-MDSC) are the three subsets of MDSCs recognized; e-MDSCs lack the myeloid lineage markers found in the first two groups[57]. It has been found that both PMN-MDSCs and M-MDSCs are linked to T cell suppression[58]. The presence of PMN-MDSCs also in the bloodstream [59] has been linked to poor survival[60] and TEX[61].

It has been observed repeatedly that RT induces MDSC expansion coupled to PD-L1 upregulation on the surface of MDSCs. This appears to occur approximately 2 weeks after RT and correlates with high levels of IL-6 and arginase activity[62,63]. In addition, the production of reactive oxygen species (ROS) is caused by RT, which dramatically activates the hypoxia-inducible factor 1 subunit alpha (HIF1α) in cancer cells[64]. As HIF1α signaling in malignant cells has been consistently associated with MDSC accumulation in the TME and enhanced immunosuppression[65], and ROS control MDSC functions[66,67], research is required to translate targeting strategies of MDSC-dependent immunosuppression into appropriate clinical scenarios, such as in patients with marked MDSC expansion/activity[68].

Regulatory T cells

Regulatory T cells (Tregs) constitute a unique subpopulation of $CD4^+$ T cells characterized by expression of the forkhead box P3 (FOXP3) transcription factor and high levels of CD25, a component of the IL-2 receptor. Tregs play a major role in restraining tumor-associated antigen-specific immune responses. It has been demonstrated that some markers, such as CTLA4, TIM3 and STAT3, play a part in the process of

mediating resistance to RT and ICB. The suppressive effects of several immune populations, including Tregs, are known to be increased by these markers [69]. It seems that activated Tregs can suppress effector cells with inhibitory cytokines, metabolic competition, or direct inhibitory action on effector T cells (Teff) and DCs by secreting inhibitory cytokines, engaging in metabolic competition or taking direct inhibitory action [Figure 2]. Such findings have been documented in HNSCC patients treated with cisplatin-based chemoradiation, where > 20% elevations in circulating MDSCs were detected and correlated with an increase in Tregs and with suppressed T cell responses[70].

Treg-APC axis. In immunologically cold HNSCC, characterized by limited T cell infiltration and poor responsiveness to RT plus anti-PD-L1 combination, antitumor immunity produced by RT can be improved through regulation of the APC-Treg axis. Treg depletion alone appears to be insufficient in tumors poorly infiltrated by Teff, which likely explains that Treg depletion combined with immunotherapy will not work. Conversely, RT is an excellent strategy to transform poorly immunogenic tumors owing to its immune-boosting properties[71]. RT plus anti-CD25 seems to be effective only in tumor models unable to induce infiltration of MDSCs, suggesting that multiple immune-suppressive populations may need to be targeted to induce a robust antitumor immune response by RT[72]. Preclinical HNSCC models have shown that treatment with anti-TIM3 plus anti-PD-L1 combined with RT[73] and anti-STAT3 plus RT[72] are potent therapeutic strategies against Tregs in appropriate tumor models. The activation of DCs and stimulation of their ability to mature and move to the lymph node have been important for eliciting a Teff response in this highly resistant HNSCC, even though this combination can effectively eliminate several tumors in animal models. In preclinical models, tumor eradication occurs when RT is coupled with anti-CD25 (Treg depletion) and anti-CD137 (DC agonism) treatment[74]. Previous studies have demonstrated that tumor necrosis factor receptor superfamily member 9 (CD137,4-1BB) can enhance DCs and reprogram Tregs[75]. Reprogramming Tregs into $Foxp3^+$ $CD4^+$ T cells with cytolytic activity was significantly aided by modulating the interaction of CD137 with its ligand. To emphasize the significance of increased antigen release for tumor eradication, it is important to note that these results were only accomplished with hypofractionated RT[74].

Cytokine responses vs immunometabolic reprogramming

Cytokines are small molecular messengers that affect immune cell proliferation, differentiation and activation, hence regulating lymphoid tissue development, immunity and inflammatory responses by controlling immune cell growth, differentiation and activation. The HNSCC TMEs are abundant in immunosuppressive phenotypes (Tregs, TAMs, MDSCs, pDCs, CAFs) which cause immune effector cells to be either excluded from the tumor or to become dysfunctional. These stromal cells not only supply the tumor cells with intermediary metabolites and nutrients, but also produce a large quantity of pro-inflammatory and proangiogenic cytokines, which together create a pro-tumorigenic environment and aid in the evasion of the antitumor immune response (see review in ref.[76]).

It is possible for tumor cells to directly secrete immunosuppressive and anti-inflammatory cytokines, such as interleukin 10 (IL-10) and TGF-β, which negatively affect APCs and T cells; other cytokines can polarize immune effector cells toward adopting an anti-inflammatory phenotype that leads to tumor progression. Expression of anti-inflammatory cytokines (IL-10, IL-6, and TGF-β) is favored in HPV-negative HNSCC. Mediating communication between tumor cells and CAFs[77] and playing a crucial role in hypoxia-induced MDSC accumulation, elevated serum IL-6 levels are linked to increased tumor burden and aggressiveness[78]. IL-10 affects the functional capacity of tumor-infiltrating pDCs, promoting the expansion of Tregs[79], and is a potential predictor of a poor clinical outcome for the treatment of HNSCCs of laryngeal origin[80]. TGF-β suppresses Teff cells and promotes Tregs[81]. Recent research suggests that a myeloid PD-L1

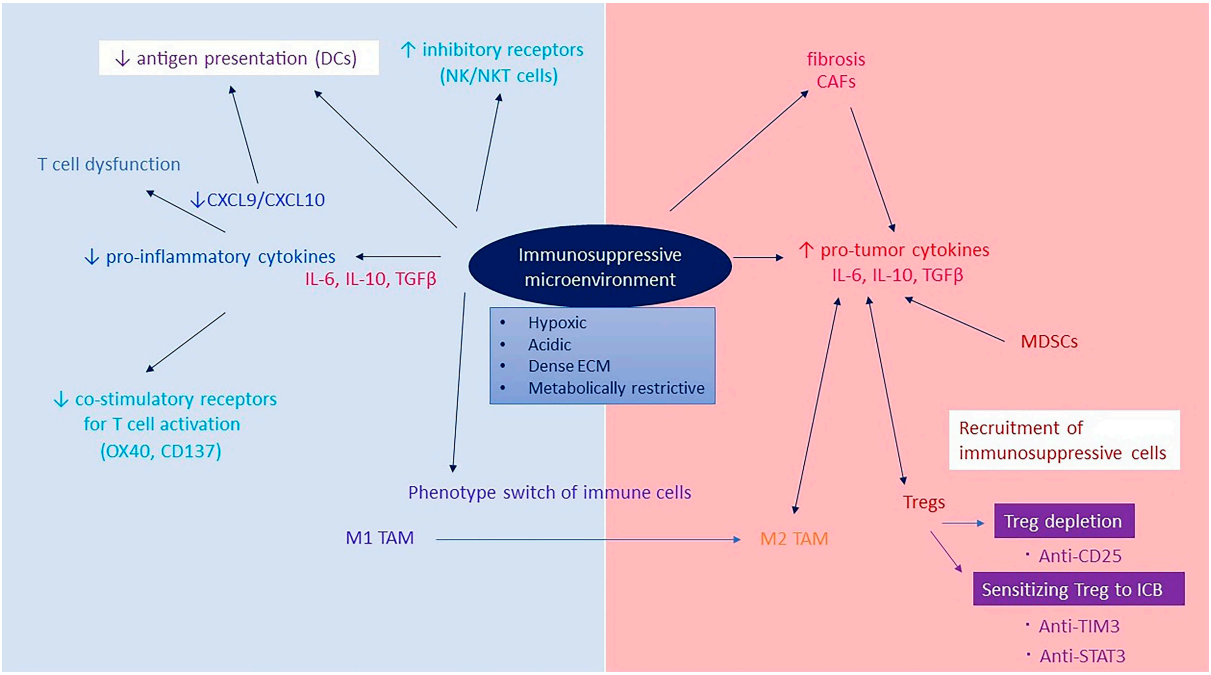

Figure 2. Immunosuppressive tumor microenvironment. Direct and indirect alterations in the TME suppress the immune response. Pro-tumor cytokines recruit immunosuppressive cells that prevent antigen presentation, which could be addressed by targeting Tregs. Reduction of pro-inflammatory cytokines generates T cell dysfunction and reduced antigen presentation. CAFs: cancer-associated fibroblasts; DCs: dendritic cells; ICB: immune checkpoint blockade; IL-6: interleukin 6; IL-10: interleukin 10; MDSC: myeloid-derived suppressor cells; TAM: tumor-associated macrophage; TGF-β: transforming growth factor beta; TME: tumor microenvironment.

blockade combined with TGF-β depletion in HPV-negative HNSCC can increase $CD8^+$ T cell infiltration in responders showing a more permissive TME for Teff function[82,83]. Thus, TME cytokine milieu-targeted therapies can either increase the production of pro-inflammatory cytokines or decrease the production of pro-tumor cytokines. More research is needed to establish the best way to manipulate local cytokines in order to modify the TME, especially in conjunction with other medications.

Chemokines are chemotactic cytokines capable of moving receptor-expressing cells along chemical gradients. The role of chemokines in cancer is conflicting in that they may facilitate the migration of tumor cells as well as attract tumor-infiltrating immune cells[84]. Patterns of chemokine/ligand-receptor expression are emerging. Growing data indicate that the CXCR3 ligands CXCL9 and CXCL10, induced by IFNγ, play an important role in immune-inflamed tumors. In contrast, even if CXCL9 and CXCL10 are present in immune-excluded tumors, the spatial exclusion of Teff cells may represent the abundance of MDSCs, which stimulate the formation of a dense stroma that restricts the T cell entry[85].

Studies of chromosome somatic copy-number alteration (SCNA, or aneuploidy) profiles have mapped the chromosomic alterations driving tumors to show reduced expression of cytotoxic infiltrating immune cells that predict response to ICB, being the strongest signals for HNSCC and pancreatic cancer[86]. In HPV-negative HNSCC, 9p21.3 loss was associated with depletion of cytotoxic T cell infiltration in *TP53* mutant tumors; and in oral cancer, 9p-arm level loss was the strongest driver of cytotoxic T cell depletion (mainly of $CD8^+$ T cells), promoting profound decreases of IFNγ-related chemokines (CXCL9, CXCL10). In addition, 9p arm-level loss and *JAK2-CD274* codeletion (at 9p24) were predictive markers of poor survival in recurrent HPV-negative HNSCC after anti–PD-1 therapy. As a consequence of the profound decrease in the chemokines CXCL9 and CXCL10, tumor-antigen cross-presentation and T cell priming and activation are

most affected since these IFNγ-inducible chemokines are known to be secreted by dendritic cDC1 cells[87,88]. Further dissection of the 9p21 and 9p24 loci credits 9p21.4 loss as the key somatic alteration to shape the immune TME response. Conversely, 9p24.1 gain may act as a driver of immune activation and ICB response in HPV-negative HNSCC[89]. Together, 9p loss promotes T cell depletion and defective IFNγ-related pathways (CXCL9, JAK2 signaling), with enrichment of suppressive cells (Tregs, MDSCs)[87,88,90]. Hence, 9p loss has been proposed as a biomarker that could better predict the clinical benefit of ICB.

The proliferation and accumulation of oncometabolites in the TME give evidence for the metabolic state in cancer. This evidence is related to the requirement to maintain aerobic glycolysis, glutaminolysis, or one-carbon metabolism.

CHANGES TO MAJOR METABOLIC PATHWAYS AND TREATMENT RESISTANCE IN HPV-NEGATIVE HNSCC

As tumors grow, metabolism in the TME switches from oxidative phosphorylation to a glycolytic pathway favoring TME acidification. According to recent research[91], metabolic plasticity in cancer cells has been demonstrated to improve antioxidant defenses and DNA repair activities, both of which can limit the efficacy of anticancer treatments [Figure 3]. In addition, the metabolic activity of cancer cells is highly plastic in response to a wide variety of environmental stresses, such as hypoxia and cytotoxic treatments, such as chemotherapy and RT. Finding new therapeutic targets for treating HNSCC could be the result of research into how ionizing radiation and other therapies affect the metabolism of cancer cells.

Oxidative stress

Radiation induces oxidative stress that damages macromolecules, and when the excess of ROS that is generated after radiation exposure remains unrepaired, the oxidative stress can be lethal. Cancer cells with strong metabolic flexibility benefit from antioxidant effects and the DNA repair metabolites provided by metabolic rewiring. So, strong antioxidant defenses are crucial to minimizing radiation susceptibility. Tumor redox metabolism was discovered as a strong predictor for radiation sensitivity in TCGA patient tumors, including HNSCC[92], showing a reliance on metabolic routes that accelerate the clearance of ROS and sustain antioxidant mechanisms as a driver of radiosensitivity. It is possible that metabolically focused therapies could modulate radiation sensitivity in this context. Numerous metabolic processes, such as glycolysis, the pentose-phosphate pathway (PPP), glutaminolysis, and one-carbon metabolism, play a role in antioxidant defenses and DNA repair. Metabolic reprogramming in cancer cells has been extensively reviewed in other publications[91,93,94].

Glycolysis and the pentose phosphate pathway

One of the main nutrients that aid in the development and survival of cancer cells is glucose. Despite the fact that glycolysis is a highly inefficient process for producing ATP, rapidly proliferating tumor cells, which require large amounts of ATP molecules due to their unsustainable rate of replication, may use glycolytic intermediaries as precursors for the anabolic pathways and biomolecules necessary for cancer cells to survive[95]. The mechanisms of glucose metabolic reprogramming vary by cancer type and sometimes even within tumors of the same origin[96].

Elevated levels of lactate and pyruvate in cancer cells drive the Warburg effect thanks to the overexpression and activation of glycolytic regulatory enzymes such as hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). In addition, the enhanced cellular transportation of metabolites through glucose transporters (GLUTs) maintains the Warburg effect. As a result of radiation, glucose metabolism is increased, which improves antioxidant activity and nucleic acid synthesis. The expression of GLUT1 has

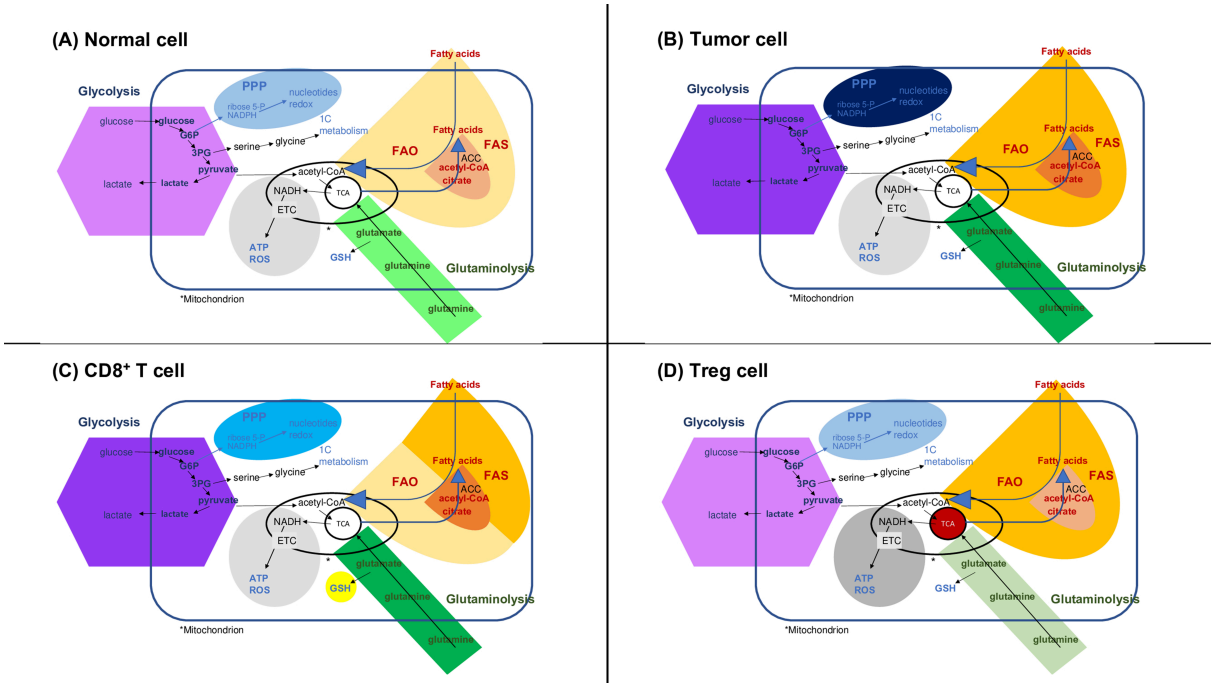

Figure 3. Metabolic pathways in (A) normal cells, (B) cancer cells, (C) $CD8^+$ T cell, and (D) Treg cell . (A): Glycolysis, the conversion of glucose to pyruvate, and the pentose phosphate pathway (PPP), that generates ribose-5-phosphate and NADPH (required for nucleotide synthesis, redox balance, and fatty acid synthesis. Pyruvate can be converted to lactate and secreted or enter the tricarboxylic (TCA) acid cycle in the mitochondria. Fatty acids can undergo fatty acid oxidation and glutamine glutaminolysis, and enter the TCA. The TCA generates NADH and FADH that can enter the electron transport chain (ETC) and contribute to the synthesis of ATP and reactive oxygen species (ROS). Citrate from the TCA can enter the cytoplasm to participate in fatty acid synthesis (FAS). Glutamine metabolism can also synthesize glutathione (GSH); (B): cancer cells increase glycolysis, lactate production, PPP, FAO, FAS, and glutaminolysis. Cancer cells also maintain certain levels of the TCA cycle and oxidative phosphorylation (OXPHOS); (C): $CD8^+$ T cells increase glycolysis, fatty acid uptake, FAS, glutamine uptake, glutaminolysis, and glutathione synthesis. The limited entry of pyruvate to the TCA favors the expression of IFNγ; (D): Tregs increase FAO, the TCA cycle, and OXPHOS; maintain PPP and glycolysis to obtain pyruvate, which feeds the increased flux of the TCA cycle and OXPHOS, while the conversion of pyruvate to lactate is restricted. Tregs limit FAS and glutamine metabolism. Increased fluxes are indicated by higher color tones.

been associated with resistance to radiation in HNSCC[97,98]. Activation of glycolysis (via HK2) and HIF1 in HPV-negative HNSCC cells has been shown to induce radioresistance[99]. The activity of glycolytic intermediates that respond to oxidative stress increases after radiation. Namely, the activity of the PPP is mediated through the activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), a master regulator of enzymes involved in ROS detoxification and elimination, via inactivation of its negative regulator Kelch-like ECH-associated protein 1 (KEAP1), which facilitates the transcription of NRF2 target genes (including metabolic genes that drive the PPP) to generate NADPH that fuels the antioxidant systems. Preclinical models have identified NRF2 as predictive of radioresistance in oral cavity SCC[100]. Moreover, the PPP can be activated via ataxia telangiectasia mutated (ATM), a redox-sensitive kinase activated in response to radiation-induced DNA damage, by inducing the activity of glucose 6-phosphate dehydrogenase (G6PDH).

Pyruvate and lactate

NAD+ is provided by the conversion of pyruvate to lactate in a highly glycolytic phenotype. Lactate production results in a requirement for lactate transport, both to prevent lactate accumulation and to provide a respiratory substrate. The temporal dynamics of the lactate-to-pyruvate ratio after radiation show a decrease shortly after radiation and increased levels over time, suggesting that redox modulation after

radiation evolves over time[91]. Monocarboxylate transporters (MCTs) are the major players in the context of lactate exchange in tumor tissue. MCT1 strongly influences lactate levels and has been suggested as a potential biomarker of chemoradiation response in HNSCC, regardless of HPV status[101]. Further studies addressing the temporal dynamics of the altered lactate synthesis and transport following radiation are required for a better understanding of associated pathways and proposal of new targets[91].

Lactate is used as a fuel[102], but it also promotes tumor growth[103]. In addition, recent findings suggest that blocking the effects of intratumoral acidity while keeping $CD8^+$ T cells' lactate metabolism at a normal level can enhance antitumor immunity[104]. For T cell function specifically, lactate appears to represent a fundamental carbon source[105] and further promotes the stemness of $CD8^+$ T cells associated with the inhibition of histone deacetylase activity[106]. A fraction of highly glycolytic HNSCC stem cells is driven by epigenetic alterations that have been found. Antioxidant defense and nucleotide synthesis are both bolstered by a gene profile associated with glutathione (GSH) metabolism and stemness in this SIRT6 loss model[107]. SIRT6 is a member of the sirtuins, a family of negative regulators of HIF1-dependent glycolysis. There is mounting evidence that increased glycolysis is a hallmark of HNSCC.

Amino acids and one-carbon metabolism

Cancer cells require amino acids as a source of energy. Next to glucose, glutamine is the most important nutrient for cancer cells since it is used in the creation of proteins and nucleic acids. It is becoming increasingly clear that glutamine is essential for metabolic remodeling in cancer under oxidative stress. One main cause for the enhanced GSH production is glutaminolysis, which is a primary source for tricarboxylic acid cycle (TCA)-derived biosynthesis[108]. This makes glutamine very necessary for ROS scavenging and anabolic needs.

One of the limiting steps in glutaminolysis is the activity of glutaminases. An emerging strategy for interfering with cancer metabolism and tumor progression is targeting glutaminolysis by inhibiting glutaminases[109]. Studies imply that targeting a specific glutaminase metabolic route is unlikely to become a successful anticancer strategy due to the TME's heterogeneity, the interconnected nature of cellular metabolism, and the plasticity of intracellular metabolic pathways. Having a better idea of which cells are using glutamine and in what pathways could help narrow down the possibilities[110,111].

The increased need for glutamine in cancer cells is met by the solute carrier (SLC) family of membrane transporters, which includes the SLC1, SLC1-6, SLC1-7 and SLC1-38 families. Emerging evidence indicates that extracellular glutamine promotes ferroptosis, an iron-dependent cell death mechanism characterized by excessive generation of lipid peroxidation, which has been shown to induce cell death through ROS accumulation in cells. Of interest, it has been recently found that radiotherapy leads to lipid oxidation and ferroptosis via repression of SLC7A11[112]. It is noteworthy that lipid membrane composition regulates radiation sensitivity, indicating that lipid metabolism may be therapeutically addressed to enhance RT efficacy. Radiation resistance and decreased RT induction of ferroptosis and lipid peroxidation are related to high NRF2 and SLC7A11 expression[113].

Nucleotide metabolism

The synthesis of nucleotides is highly required for DNA repair of double-strand breaks after radiation damage, especially in less radiosensitive cell types[94]. Consequently, an efficient DNA damage response (DDR) is linked to radiation resistance in cancer cells. Alkylating agents, such as cisplatin and lipid peroxidation resulting from ROS accumulation, can generate DNA adducts that also result in metabolic reprogramming through all major pathways (reviewed in ref.[114]). *De novo* synthesis of nucleotides, which

affects DNA replication and repair, requires certain metabolites, such as glutamine and aspartate. The DDR's activity is affected by cellular metabolism because of changes in substrate availability. The DDR is capable of regulating metabolic pathways that cause or protect against DNA damage, as well as those that rearrange chromatin and are necessary for DNA repair[115].

Signaling via purine nucleotides and nucleosides, such as adenosine and adenosine 5′-triphosphate (ATP), is increased in HNSCC[116]. The balance between ATP (pro-inflammatory) and its catabolite, adenosine (anti-inflammatory), is tightly controlled in immune microenvironments at multiple levels. Adenosine signals in DCs upregulate IL-10, TGF-β, and arginase-2, promoting tumor growth. In TAMs, adenosine induces pro-tumor M2 macrophage polarization by reducing the expression of interleukin 2 (IL-2), tumor necrosis alpha (TNFα), and nitric oxide but upregulating arginase-1, IL-10, and vascular endothelial growth factor (VEGF)[117]. However, the balance is shifted to increased adenosine production in tumors, rendering a deeply immunosuppressed TME[118]. Moreover, ATP is rapidly converted into adenosine in the TME by the ectonucleotidases CD39 and CD73, which are particularly expressed in $CD25^+$ or $FoxP3^+$ Tregs. Hypoxia, via HIF1α, upregulates CD39 and CD73, further enhancing the adenosine pathway via adenosine receptor A2A (A2AR) in HNSCC[119,120]. Cancer cell death induced by RT releases ATP dose-dependently, which activates DCs and triggers an antitumor immune response. Conversion of ATP to adenosine can be generated directly by RT, via production of ROS, which activates TGF-β and promotes the M2 TAM phenotype[121]. Therapeutic targeting of A2AR, CD73, and TGF-β may shift the TME to a pro-ATP environment and reduce resistance to immunotherapy in the setting of RT. Purinergic signaling stands out as a particularly promising target for the development of novel anticancer agents, for the most part in combination with standard-of-care therapeutics or ICB[122,123].

Fatty acids and lipids

Lipid metabolism is an area of cancer metabolic reprogramming that has received less attention. The need for metabolic intermediaries in macromolecule production is particularly great in cancer cells. In order to produce membrane-forming, energy-storing, signaling-molecule-generating and ATP-generating molecules, lipid metabolism is a crucial route. Alterations in lipid availability are associated with altered cancer cell motility, angiogenesis development, metabolic symbiosis, immune surveillance evasion, and treatment resistance. The reprogramming of fatty acid (FA) metabolism in tumor tissues has gained considerable interest as a potential cancer treatment target[93]. This suggests that FAs may be another environmental resource that $CD8^+$ Teff cells need to compete with tumor cells in the TME[124], given that $CD8^+$ Teff cells also take up FAs at high rates.

The first stage in FA metabolism is for FA transporters (CD36/FAT, FABPpm, and FATPs) to bring FAs into the cell. In order to power keratogenesis and the TCA cycle, mitochondria convert imported FAs into fatty acyl-CoA before transporting it for oxidation. Lipogenesis enzyme overexpression, FA trafficking, and FA oxidation (FAO) are among the ways in which FA metabolism can be influenced. Key regulators of lipogenesis include stearoyl-CoA desaturase 1 (SCD1), fatty acid synthase (FASN), acetyl-CoA carboxylase (ACC), and the sterol regulatory element-binding proteins (SREBPs). There is a class of proteins called FA binding proteins (FABPs) that play a role in the transport of FAs within cells. Cancer cells rely heavily on FAO reprogramming. FAO generates cytosolic NADPH to assist biosynthesis and can produce twice as much ATP as carbohydrates.

Studies on FAs in patients with HNSCC are limited. Abnormal elevation of the FA transporter CD36 in oral HNSCC promotes tumor metastasis. In contrast, its inhibition leads to complete remission or elimination of lymph node and lung metastases in *in vivo* models[125]. In fact, acidosis-induced TGF-β2 activation

stimulates both partial epithelial-to-mesenchymal transition and FA uptake via CD36 that are stored in lipid droplets (LD). LDs represent energy stores for cancer invasion and dissemination[126]. The lipogenesis enzyme FASN was shown to promote radiation resistance in preclinical HNSCC models[98].

There is a correlation between radiation, lipid redox, and ferroptosis that has recently been established in the literature. Liposomes undergo peroxidation and lipid fragmentation after radiation, while radiolysis generates oxygen radicals that cause lipid peroxidation of polyunsaturated FA (PUFA), in a radiation dose-dependent manner[127]. The excess of lipid oxidation leads to ferroptosis. Ferroptosis is a form of cell death induced by iron-dependent lipid peroxidation. Ferroptosis induction is dictated by the proportion of PUFA in the lipid membrane[128]. Thus, targeting lipid metabolism may accentuate the effect of radiation. Upregulation of SLC family members that regulate cystine import (critical for GSH biosynthesis and maintenance of the antioxidant pool within the cell) has been related to acquired RT resistance. The suppression of SLC7A11 by RT-activated ATM results in decreased cystine absorption, enhanced tumor lipid oxidation and ferroptosis, and improved tumor control[112]. Additionally, low levels of RT-induced ferroptosis and lipid oxidation are associated with high levels of NRF2 and SLC7A11 expression, which is linked to resistance to RT[113]. In HNSCC, preclinical models suggest that NRF2 inhibition could restore cisplatin sensitivity[129]. In another model, the inhibition of the glutamine transporter SLC1A5, expressed at high levels in the CD44 variant-cancer stem-like cells ($SLC1A5^{+}/CD44v^{high}$), triggered oxidative damage[130]. Taken together, these findings show that cystine transporters represent an original and targetable mechanism to improve RT efficiency.

It has been hypothesized that PMN-MDSCs in the TME of HNSCC use ferroptosis as an immunosuppressive mechanism. Even though ferroptosis reduces PMN-MDSC numbers in the TME, it inhibits T cell function by increasing the release of immunosuppressive chemicals. It has been postulated that ferroptotic PMN-MDSCs exert suppression by soluble substances, possibly including prostaglandin E2, and is favored under hypoxic settings, in contrast to their main mechanism in peripheral lymphoid organs, which is direct interaction between PMN-MDSCs and T cells[131]. Additional work is required to understand how to integrate ferroptosis into the clinical setting.

Role of hypoxia

Rapid tumor cell proliferation in the TME creates low-oxygen regions with uneven concentrations of oxygen, leading to hypoxic stress. Extreme low oxygen levels stimulate a tolerant TME that allows evasion of immune surveillance[132]. In particular, the hypoxic TME encourages the formation and production of immunosuppressive cells and secretion of substances (e.g., VEGF and TGF-β), and upregulation of immune checkpoint molecules on cancer cells to limit the capacity of effector immune cells to eradicate cancer cells. Under hypoxic conditions, the tumor ECM stimulates collagen synthesis and maturation, thereby generating a highly dense ECM[133]. The stabilization of HIF-signaling by hypoxic cells alters cellular redox and increases the use of glycolysis and alternate metabolizing pathways, which in turn increases resistance in the surrounding cells[134].

In contrast, hypoxic signaling may trigger angiogenesis, which will cause reoxygenation of tumor tissue, and enhanced radiosensitivity. In spite of this, there has been evidence that radioresistance persists after reoxygenation of hypoxic cells caused by G1 arrest/quiescence. These findings may suggest that tumor cells in chronic hypoxic regions, which are often located near necrotic zones due to an inadequate oxygen supply[135], acquire a quiescent state[136] and that reoxygenation alone is not sufficient to re-sensitize previously hypoxic cells. Notably, in cases of HPV-positive HNSCC, this condition disappears entirely. This would suggest that hypofractionation may maximize cancer cell death, while the interfraction interval

should be tuned to provide optimal re-entry of previously hypoxic cells into the cell cycle[19].

It has been shown that HNSCC patients with persistent tumor-associated hypoxia during chemoradiation had a worse outcome[137] linked to high levels of tumor-infiltrating lymphocytes[138], highlighting the clinical relevance of persistent hypoxia in HNSCC.

BRIDGING THE GAP

The local TME of the tumor and T cell priming in the lymph nodes greatly define the potency of the cytotoxic immune response[139]. DCs play an essential role in $CD8^+$ T cell differentiation and antitumor activity[140]. Preclinical research to exploit these traits[25,74,141] has been clinically translated recently, offering new strategic opportunities in HPV-negative HNSCC. In addition, considering the more active role of tumor-draining lymph nodes merits consideration.

Innovative preclinical models to evaluate the TME in HNSCC

Apart from animal models, other tools have been incorporated to account for the effect of immune infiltration in the TME. In the context of personalizing treatment options, major efforts are being directed towards the development of reliable three-dimensional experimental models to study the microenvironment and treatment-resistance mechanisms.

Amongst them, organotypic co-culture models to culture fresh HNSCC tumor explants allow cultivation of cancer slices for up to 21 days in their original tumor microenvironment and investigation of the clonal expansion of resistant cancer cells upon treatment[142]. A lymphatic organotypic microfluidic model has been proposed to study lymphangiogenesis, consisting of a lymphatic vessel surrounded by primary tumor-derived fibroblasts, which offers the potential to evaluate HNSCC metastasis to lymph nodes via lymphatic vessels accounting for the heterogeneity of the individual TME[143]. An alternative system is a biomimetic collagen-based scaffold, which has been used to study the impact on the phenotype and genotype of oropharyngeal cancer cells. The 3D architecture in this model enables the study of the induction of migration properties and of the expression of epithelial-mesenchymal transition markers [144].

One emerging field uses microfluidic platforms to study the TME and how immune cells and tumor cells interact with it. Organs-On-Chip are innovative tools that have boosted the research on cell-cell interactions and migratory behaviors. In the oncoimmunology field, these platforms are called OncoImmuno chips, and in their most ambitious objective aspire to become a Human-On-Chip (HOC, a multicellular chip module that contains all the key cellular components and extracellular factors derived from a specific human donor's immune cells)[145]. In this regard, a HOC recapitulating the systemic metastatic spread has been proposed, composed of different modules that in the near future could be connected to generate a working metastasis HOC[146].

Clinical translation

Because immunological dysfunction can develop over time and while treatment is being received[147], the optimal timing of immunotherapeutic intervention, such as neoadjuvant therapy[148], window of opportunity trials[149] for surgically resectable HNSCC, concurrently with standard definitive treatment, or as adjuvant or salvage therapy, has yet to be determined.

Patients with HPV-negative HNSCC who participated in a recent phase I/Ib trial and were treated with neoadjuvant hypofractionated stereotactic body radiotherapy (SBRT) with a single dose of durvalumab had an overall survival of 80.1% (95% CI, 62%-100%), locoregional control/progression-free survival of 75.8%

(95% CI, 57.5%-99.8%), and major/complete pathological response of 75% (95% CI, 51.6%-100%)[150]. It has been demonstrated that combining immunotherapies with RT can boost the infiltration of immune cells into the TME, and immunological priming is the intended outcome of this research. It has been demonstrated that SBRT is able to increase antitumor immune function by increasing the abundance of T cells in the TME and activating those T cells. A single dosage of the neoadjuvant durvalumab is administered to patients anywhere from three to six weeks prior to the scheduled standard operation. This dose was provided concurrently with neoadjuvant SBRT to regions that exhibited clear signs of disease. This was escalated using a 3 + 3 model. Initially, adjuvant therapy based on pathology was used, but in the expansion cohort, none of the patients who achieved pathological major or complete response received adjuvant treatment[74]. All of the patients received adjuvant durvalumab between 6 and 12 weeks after their surgeries, with a maximum of 6 doses per patient. It was concluded that a total of 24 Gy, split up into three doses, is the maximum dose of SBRT that can be safely given. The maximal pathological response was not seen until at least 5 weeks after radioimmunotherapy was finished. This likely indicates the amount of time required to create systemic immunological memory. Specifically, a biological correlation research study found that patients who had a positive response to treatment had higher levels of Teff cells, lower levels of immunosuppressive cells, and improved antigen presentation. The changes that occurred in the draining lymph nodes (DLNs) of responders provided evidence for enhanced antigen presentation and T cell priming in the DLNs. This was demonstrated by an indication of a shorter distance between DCs and T cells. It has been observed that when $CD8^+$ T cells were located closer to cancer cells in the TME, outcomes were much better than using the overall number of $CD8^+$ T cells[151]. The findings of this research suggest that the two patterns are related.

In a different trial, a phase Ib/IIa study testing neoadjuvant combined checkpoint blockade prior to surgery in HNSCC, two doses of nivolumab plus one dose of ipilimumab were administered 4 weeks before conventional surgery, and resulted in 35% of major pathological response[152]. Notably, the response was discordant between primary HNSCC and its lymph node metastases. As it might be a matter of time to surgery, other potential explanations might apply.

A role for tumor-draining lymph nodes in immunotherapy

A lymph node has been found to have a lipid-rich milieu that tumor cells may preferentially utilize as an energy source[153]. Moreover, new evidence suggests that FAO can aid in the settling of circulating tumor cells (CTCs) in lymph nodes[154]. This lymph node pre-metastatic niche (PMN) requires lymphangiogenesis, recruitment of immunosuppressive cells, upregulation of chemokines and cytokines and vascular remodeling[155]. Proliferation and differentiation of lymphatic endothelial cells cause lymphangiogenesis, which has been demonstrated to be regulated by FAO[156]. Tregs, MDSCs, TAMs and pDCs within the PMN potentially suppress antitumor immune responses. However, Teff cells, being at a disadvantage in competing for glucose with tumor cells, redirect their metabolism towards FAs. Furthermore, Tregs may augment FAs, thus influencing effector cell development. FAO dominates in M2 TAMs, favoring interleukin 1 beta (IL-1β) secretion and tumor cell migration[157]. MDSCs activate FAO enhancing the ability to suppress T cell function. Recent studies show that stromal and immune cells in the PMN secrete substances in concert with FAO, including TGF-β and vascular endothelial growth factor-C (VEGF-C)[158]. Vascular remodeling, also known as tumor cell-induced microvascular expansion, may increase angiogenesis and vascular permeability to promote metastasis[159]. As a result of an increase in HIF1 and hypoxia-induced proangiogenic factors, CTCs from the vasculature and lymph vessels may reach the PMN and recruit more tumor cells. Modifications to the microenvironment of the lymph node, such as lymphangiogenesis, an increase in the expression of immunosuppressive cytokines, and an increase in the number of immunosuppressive cells, make it possible for tumor cells to thrive and remain dormant until stimuli drive their progression into a micrometastasis.

The potential role of the lymphatic system in driving immune evasion and metastasis has become highly relevant in cancer research[26]. The state of the TME may become functionally altered depending on lymphatic activation states. In support of the hypothesis that lymph node metastasis is a functional driver of disease, a recent publication finds that metastatic lymph nodes display Treg expansion that generates tolerance to tumor cells and enhanced metastatic potential[160]. The effect of increasing tumor burden remains to be determined. There is evidence of a progressive loss of T cell proliferation accompanied by the proliferation of Tregs[27]. Whether this hypothetical setting can influence the established role of complete lymphadenectomy, which has shown limited survival impact, or provide an opportunity for neoadjuvant immunotherapy to target and remodel regional lymph nodes, remains to be seen. An improved understanding of lymphatic adaptation in tumor progression and response to immunotherapy could lead to more effective treatment strategies.

CONCLUDING REMARKS

The multiplicity of immune evasion mechanisms that immune-cold tumors can adopt is a major barrier to benefit from immune therapy strategies. The impact of immunotherapy in HPV-negative HNSCC has been rather low, indicating that a better understanding of the immune escape mechanisms in these tumors can definitively enhance their immunogenicity and treatment response. This review proposes that modulation of the TME to relieve immunosuppression, creation of a metabolically permissive TME, and priming the immune system are promising strategies to remodel the TME to overcome treatment resistance in HPV-negative HNSCC [Figure 4]. However, the concepts that have been discussed have yet to be implemented in the clinic, reflecting the preliminary stage of this information. Nonetheless, a few salient points can be summarized.

Evidence that an efficient antigen presentation machinery elicits a potent immune response in HNSCC has been studied in two phase I-I/II trials[150,152] with strong accompanying correlative studies. Major or complete pathological responses of 75% were shown after neoadjuvant SBRT to gross disease and a single dose of durvalumab, followed by adjuvant durvalumab of up to 6 doses[150]. The biological correlates indicated that response was related to higher levels of Teff cells, lower levels of immunosuppressive cells, and improved antigen presentation and T cell priming in the draining lymph nodes. Of note, when DCs and T cells were closer in the draining nodes, and $CD8^+$ T cells and tumor cells were closer in the TME, outcomes were better[151]. In preclinical models, Treg depletion or reprogramming strategies in combination with radiation have proven to activate DCs[72-74]. A central tenet of this strategy is the use of SBRT only on gross disease, which optimizes the presence of neoantigens and allows a better coordinated immunologic response in the draining lymph nodes. Additional data confirmation and a refined consideration of the different contributions of the immunosuppressive cells that compose the heterogeneous TME will further guide the strategic scope of this treatment approach.

Robust data exist that indicates that the functionality of the IFNγ-inducible chemokines CXCL9 and CXCL10, essential for the recruitment of $CD8^+$ T cells and NK cells, favors T cell trafficking and infiltration. Multiomic analyses of HPV-negative HNSCC cohorts have identified that losses at genomic regions on the chromosome 9p containing IFNγ-pathway genes result in $CD8^+$ T cell depletion and CXCL9/CXCL10 suppression, and predict immune-cold, ICB-resistant tumors[87-89]. Pan-cancer studies suggest that this mechanism is prominent in HNSCC and pancreatic cancer[86], and has become a biomarker that synergizes with PD-L1/TMB for patient stratification. Further genomic/non-genomic dissection of these alterations can provide new strategies to target these tumors.

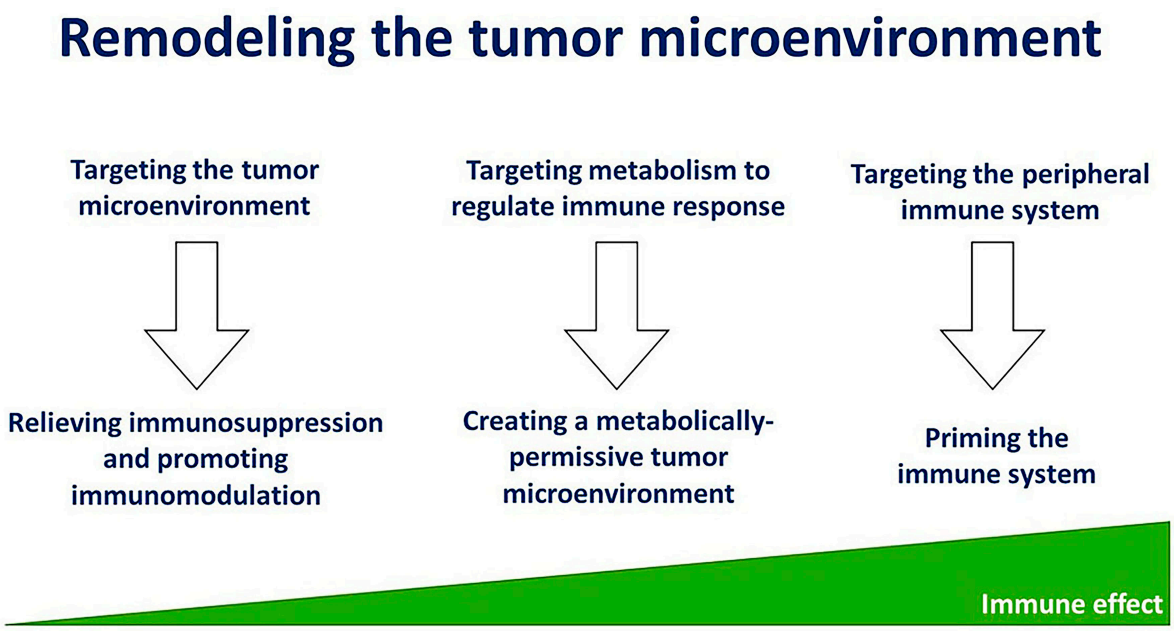

Figure 4. Immunomodulation, creating a metabolically permissive microenvironment, and priming the immune system to remodel the tumor microenvironment and elicit a strong immune response.

Studies evaluating the metabolic alterations that occur in the TME have uncovered potential treatment targets, like the transcription factor NRF2 which supports the PPP[100]. Preliminary data suggest that the amino acid cysteine may play an important role in mediating $CD8^+$ T cell-induced ferroptosis[161], a recently identified form of regulated cell death. Ferroptosis, the mechanism by which abnormal intratumoral lipid metabolism induces cell death, has attracted great attention as a potential novel target in oncology[162]. The discovery that radiotherapy induces ferroptosis in cancer cells as a result of an ATM-mediated downregulation of SLC7A11, and that this effect is enhanced in combination with ICB in animal models, has evolved into an ongoing active area of investigation[112].

In conclusion, while additional research is needed, confidence exists that well-designed preclinical and clinical studies to assess neoadjuvant SBRT schedules in combination with immunotherapy, the clinical applicability of 9p loss, and the role of ferroptosis in cancer will elucidate which patient subgroups benefit the most. A better definition of contributing factors of an immunogenic microenvironment constitutes a significant step forward, which could be further exploited by incorporating emerging factors like genomics or the influence of the microbiome[163].

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Authors' contributions

The author contributed solely to the article.

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Cancer Drug Resistance

Review

Open Access

Revisiting mechanisms of resistance to immunotherapies in metastatic clear-cell renal-cell carcinoma

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Abstract

Renal-cell carcinoma (RCC) remains a leading cause of cancer-related mortality worldwide. Though newer therapeutic combinations of immune checkpoint inhibitors and targeted therapies have greatly improved outcomes, resistance to these therapies is becoming a challenge for long-term control. Mechanisms of resistance have been explored in a variety of solid tumors, including RCC. Based upon our review of the current literature on the mechanisms of resistance to immunotherapies for the management of metastatic clear-cell renal cell carcinomas (mccRCC), the ensuing conclusions have been made:

The management of mccRCC has progressed substantially with the advent of checkpoint inhibitors and targeted oral therapies, alone and/or in combination.

Nevertheless, innate or developed resistance to these therapies remains an ongoing challenge, particularly to immune checkpoint inhibitors (ICIs).

Several of the known mechanisms of resistance have been well defined, but recent progression in cellular therapies helps to expand the armamentarium of potential combination options that may overcome these modes of resistance and improve long-term disease control and survival for an otherwise dismal disease.

In the ensuing review and update of the literature on the mechanisms of resistance to immunotherapies in mccRCC, we have revisited the known resistance mechanisms of immunotherapies in metastatic clear-cell RCC and explored ongoing and future strategies to overcome them.

Keywords: Metastatic clear-cell renal-cell carcinoma, immune therapy, checkpoint inhibitor resistance, chimeric antigen receptor T-cell therapy

INTRODUCTION

Renal-cell carcinoma (RCC) remains a common cause of morbidity and mortality worldwide. It is the eighth most common cancer diagnosed in the United States, and in 2022 there were an estimated 79,000 new cases diagnosed, accounting for 4% of new cancer diagnoses in the country[1]. Though the relative 5-year survival is nearly 77%, the prognosis of advanced RCC remains dismal with an estimated 5-year survival of 15%[1]. Clear-cell renal-cell carcinoma (ccRCC) still accounts for the majority of RCC, with non-clear-cell histologies making up about 25%[2].

Historically, standard chemotherapy and radiation have been ineffective for ccRCC. Clinical studies suggested an immunologic role in the growth and control of RCC, particularly the presence of tumor-infiltrating lymphocytes (TILs) within the tumor and the process of immune evasion[3-5]. Immune therapies were then explored as potential therapeutic options, particularly immune cytokines such as interferons and interleukin-2 (IL-2) and later immune checkpoint inhibitors (ICIs). A better understanding of the role of programmed death 1 (PD-1), programmed death ligand 1 (PDL1), and cytotoxic T-lymphocyte–associated protein 4 (CTLA4) led to the eventual use of ICIs in metastatic renal-cell carcinoma (mRCC).

The discovery of a defective von Hippel-Lindau (VHL) gene as a major molecular alteration in the pathogenesis of ccRCC was another leap forward. VHL alterations resulted in upregulation of several growth factors involved in angiogenesis (platelet-derived growth factor receptor-beta, vascular endothelial growth factor [VEGF], and transforming growth factor alpha) using the hypoxia-inducible factor (HIF) pathway. This led to the development of newer therapies specifically targeting these factors[6]. VEGF-receptor tyrosine-kinase inhibitors, such as sunitinib, pazopanib, and sorafenib, quickly became the standard of care given their improved response rates, more convenient administration, and manageable toxicity profiles[7-9].

Single-agent use of tyrosine-kinase inhibitors and ICIs was effective but with limited responses and long-term control. Resistance, particularly to ICIs, remains a barrier to achieving and maintaining a durable response to these therapies. It is also important to note that ICIs have several potential adverse effects which must be taken into consideration, especially with long-term use[10]. Efforts have been made to combine immunotherapies and anti-angiogenic agents with each other and with other drugs. Other therapies include chemotherapy and radiation for potential synergistic and immunomodulatory effects and to overcome this resistance. Recent reviews summarized potential mechanisms but primarily focused on anti-angiogenic drug resistance[11]. In a prior review by Moreira *et al.* published in this journal, resistance mechanisms to immunotherapies in the management of metastatic RCC were explored[12]. Here, we revisit these mechanisms and discuss updated ongoing and future strategies for overcoming resistance, particularly adoptive cellular therapies.

Aim

To review and update the literature on mechanisms of resistance to immunotherapies in mccRCC.

Methods

Various internet databases were searched, including: PUBMED, Yahoo, Google, and Google Scholar. The search words that were used included: metastatic clear-cell renal-cell carcinoma, immune therapy, immune checkpoint inhibitor, immune checkpoint inhibitor resistance, and chimeric antigen receptor T-cell therapy. One hundred and six (106) references were identified, which were used to write the review and update the literature on the mechanisms of resistance to immunotherapies in mccRCC.

CURRENT THERAPEUTIC LANDSCAPE

The current treatment landscape for ccRCC in the first and second lines reflects the efficacy seen with immune and anti-angiogenic agents, both alone and in combination, over previously standard cytokine therapies. Choice of treatment is in part led by risk as determined by the International mRCC Database Consortium and Memorial Sloan Kettering Cancer Center/Motzer risk-stratification criteria[13,14]. Individual patient factors, including comorbid conditions, concomitant medications, and socioeconomics, also play a large role in the choice of therapy.

Current preferred first-line treatments for favorable risk metastatic ccRCC include: pembrolizumab/axitinib, nivolumab/cabozantinib, and pembrolizumab/lenvatinib, with response rates ranging from 55% to 71%[15-17]. Current preferred first-line treatment options for intermediate/poor-risk disease include: pembrolizumab/axitinib, nivolumab/cabozantinib, nivolumab/ipilimumab, pembrolizumab/lenvatinib, and cabozantinib[18].

Second-line treatments vary and include anti-angiogenic and immune-therapy agents that the patient may not have previously received. Inhibitors of the PI3K/AKT/mTOR pathway, such as everolimus and temsirolimus, currently have a role in later lines of therapy[19,20].

MECHANISMS OF RESISTANCE

ICIs have now become essential in the therapeutic armamentarium for several malignancies, including urothelial carcinoma, melanoma, and non-small-cell lung cancer. In addition to approved combinations for metastatic ccRCC, ICI was recently integrated into the adjuvant treatment of ccRCC with the approval of pembrolizumab post-nephrectomy for high-risk disease, based on results from KEYNOTE-564, noting a disease-free survival benefit of nearly 10% at 24 months, and 30 months disease-free survival HR of 0.63[21,22]. Though ICIs have been very effective, we now see evidence of resistance to these therapies, which limits the durability of response.

The complex and intricate interaction between the immune system and the cancer cell has been described by the various host- and tumor-specific characteristics that have an impact on this interaction and are visually depicted by Blank *et al.*, where potential and confirmed biomarkers at these levels are also noted [Figure 1][23]. However, this interface is complex, and many parts of this interaction remain undiscovered. In general, immune resistance can be innate, acquired, intrinsic, or extrinsic[24]. Innate or primary resistance is an immediate lack of response caused by the presence of resistant clones before starting treatment. Acquired or secondary resistance occurs while on active therapy after an initial response to treatment. Intrinsic resistance occurs when the tumor cells interfere with internal processes, such as cell signaling, gene expression, DNA damage response, and immune recognition, whereas extrinsic resistance occurs through T-cell activation and other processes outside the cell[24]. Moreira *et al.* divided potential factors contributing

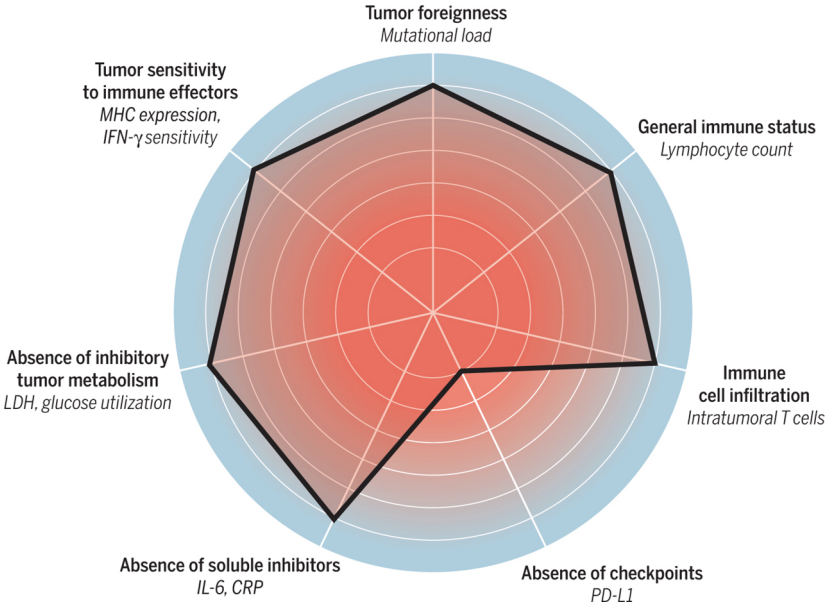

Figure 1. The cancer immunogram - parameters that characterize aspects of cancer-immune interactions where biomarkers have been or may be identified[23]. Reprinted with permission from the American Association for the Advancement of Science.

to resistance into three major groups - patient, tumor cell, and tumor microenvironment (TME)[12].

Patient-associated factors of resistance

Patient-related factors include sex/gender, HLA genotype, sarcopenia, gut microbiome, and antibiotic and corticosteroid use. Recent data have continued to show trends toward sex-related differences in response to ICI, favoring males over females[25-27]. Over recent years, there has been tremendous interest in the relationship between the gut microbiome and ICI response. Several studies in melanoma and renal-cell carcinoma have shown that increased diversity in intestinal bacteria is associated with better responses to checkpoint blockade and that certain species may improve or diminish response[28,29]. Mouse-model studies exhibited that the microbiome may alter the amount of tumor dendritic cells, antigen-presenting cells, and cytokines[30]. Species associated with improved response in mRCC include *Akkermansia* spp. and *Bifidobacterium* spp[28]. In a recent phase I study, Dizman *et al.* investigated the use of nivolumab and ipilimumab with or without CBM588, a bifidogenic live bacterial product, in mRCC and found enhanced outcomes among patients receiving combination therapy with the probiotic agent[31]. Moreover, antibiotic therapy may alter ICI response because it dysregulates the microbiome. Studies found poor ICI response associated with antibiotic use among patients with mRCC on immunotherapy[32]. This was also evaluated in a recent meta-analysis of 10 studies which found decreased progression-free survival (PFS), overall survival (OS), and objective response rate (ORR)[33]. In a recent population-based study of patients over 65 years of age, antibiotic use within 1 year of ICI therapy, in particular fluoroquinolones, was associated with worse OS[34]. Additionally, steroid use while receiving ICIs has been associated with poor outcomes (PFS and OS). In a recent meta-analysis of 16 studies including non-small cell lung cancer and melanoma, steroid use for supportive care or brain metastases was associated with reduced OS rather than use for adverse effects from ICIs[35]. Ongoing studies are evaluating ways of modifying the gut microbiome and factors of antibiotics and steroid use that may be modifiable and affect response to therapy.

Tumor cell-associated factors of resistance

Tumor cell-related factors for immune evasion and resistance include altered methods of antigen presentation and T-cell exhaustion. By reducing the expression of tumor antigens and downregulating MHC class I, tumor cells may avoid immune surveillance and destruction[36]. Alternatively, chronic exposure to an antigen may lead to upregulation of PD-1 or the expression of other inhibitory receptors, such as TIM3, LAG3, BTLA, TIGIT, and VISTA[24,37,38].

This interaction between tumor antigens and immune response led to the theory of combining immunotherapies and stereotactic body radiation therapy (SBRT) as a therapeutic option. Radiation causes tumor necrosis and may release more tumor antigens, which in turn may allow ICIs to work more effectively both locally and potentially beyond what is irradiated. Several studies evaluated the efficacy of this approach, including NIVES, RADVAX RCC, and a high-dose IL2 + SBRT study. NIVES was a phase II study evaluating the role of nivolumab and SBRT in a pretreated patient with mRCC. Sixty-nine patients were enrolled with ORR of 17%, disease control rate of 55%, and OS of 20 months. However, the authors concluded that this combination did not result in improved outcomes among pretreated patients but could be studied further and considered in an oligometastatic population[39]. RADVAX RCC evaluated dual-checkpoint blockade with ipilimumab and nivolumab and SBRT. Twenty-five patients were enrolled and initial analysis noted an ORR of 56%[40]. Interleukin-2 with SBRT has been studied in metastatic melanoma and mRCC with promising antitumor activity[41,42].

TME-associated factors of resistance

The TME includes factors extrinsic to the cancer cell and plays a substantial role in regulating T-lymphocytes. The balance between Tregs and Teff cells is an important factor in the response or resistance to ICI, with greater Tregs resulting in a diminished response. Moreover, myeloid-derived suppressor cells are another regulatory mechanism that allows for continued tumor growth through immune regulation[24]. Tumor-associated macrophages, TGF-beta, VEGF, and cytokines are all involved in these regulatory processes and may alter the response to ICI therapy.

Chronic antigen exposure can result in upregulation of PD-1 expression and ultimately T-cell exhaustion, a hypofunctional state associated with decreased Teff function[43,44]. Exhausted T cells in cancer are similar to those in chronic viral infections, and upregulation of immune checkpoints is a hallmark feature[45]. This exhaustion in turn may alter antigen presentation and can be a potential mechanism of resistance to checkpoint blockade in multiple tumor types, including mRCC[46-48].

TILs are strong tumor-defense mechanisms regulating growth and spread. In most malignancies, higher amounts of TILs have been associated with better prognosis and response to ICIs[49-51]. However, there are some tumor types in which more TILs are not necessarily better. Though RCC is a heavily T-cell - enriched tumor with high numbers of CD8+ TILs, these are mostly dysfunctional or exhausted[52-55]. They also express more inhibitory receptors, such as LAG3 and Tim-3, co-expressed with PD-1, which have been associated with more aggressive phenotypes exhibited by higher TNM staging and a higher Fuhrman grade[49,56].

Hypoxia is a major feature in the TME of RCC that may contribute to immune dysregulation and tumor progression through several different mechanisms, as was previously described by Moreira *et al.* and others[12,38,57,58]. Hypoxia results in the release of hypoxia-inducible factors 1 and 2 (HIF-1a and HIF-2a), which stimulate the expression of inhibitory signals, such as VEGF, CTLA4, and LAG3, and suppress T-cell activation and function[12]. Belzutifan, a selective HIF-2a inhibitor, was studied in a phase I trial with promising antitumor activity in heavily pretreated patients with mccRCC[59]. It is currently FDA-approved for VHL disease - associated tumors, including RCC, CNS hemangioblastomas, and pancreatic

neuroendocrine tumors[60]. The use of belzutifan in other settings of RCC and in combination with other therapies, including ICI, is under investigation. Tumor-intrinsic factors and the TME and their interplay are illustrated in Figure 2 as originally developed by Ballesteros *et al.*[61].

FUTURE DIRECTIONS

Proposed strategies to overcome resistance to ICIs include novel combination approaches, including checkpoint inhibitors with cytokine or chemokine therapy, adoptive cellular therapies, or oncolytic viruses. Biomarkers remain elusive in mRCC as most, including PD-L1, have failed to predict therapeutic response[62-64]. Cell-free circulating tumor DNA (ctDNA) may have some utility in mRCC but is limited by lower levels in RCC compared to other solid tumors[65,66]. Those with higher tumor volume appear to shed more ctDNA[67]. Tumor mutation burden may also be a useful predictor of response to ICIs because a higher tumor mutation burden is associated with a favorable response, though this does not appear to hold for combined checkpoint inhibitors[68]. Recently, transcriptomics, metabolomics, and metabolic profiling of RCC cells have allowed for a potential avenue of tumor and resistance detection based on common metabolic features of the cancer cell. Lower OS and greater ICI resistance have been noted in tumor cells with an altered kynurenine/tryptophan ratio and higher hypoxia and Wnt/beta-catenin signaling[69-72].

Revisiting cytokine therapy

The success of ICIs is limited by resistance reinvigorated interest in cytokine therapy, which had originally been effective in RCC, though without robust responses (around 20%) and their use was limited by challenges in administration and potential toxicities[73-75]. In a retrospective analysis, Buchbinder *et al.* evaluated response among patients with metastatic melanoma and mRCC who had received HD IL-2 after prior treatment with PD-1/PD-L1 therapies through the PROCLAIM database. Of the 57 total patients, 17 had mRCC and ORR was 24% with two complete responses[76]. In the PIVOT-02 phase I/II study, bempegaldesleukin, a pegylated form of IL-2, was tested with nivolumab as the first line in mccRCC. Initial tumor activity was noted with an ORR of 35% and a complete response of 6%[77]. This is being evaluated further in PIVOT-09, the phase III study, though enrollment was recently terminated as of November 2022[78,79]. Another recent early-phase trial studied pembrolizumab with HD IL-2 in mRCC and noted an ORR of 70% and no worsening toxicities suggesting further exploration[80].

Chimeric antigen receptor T-cell (CAR-T) therapy

CAR-T therapy is an adoptive cellular treatment that has revolutionized the management of refractory hematologic malignancies. In essence, a patient's T cells are removed and modified against a specific cellular target and then infused back into the patient (autologous therapy)[81]. This began with the FDA approval of axicabtagene ciloleucel (axi-cel), an autologous anti-CD-19 CAR-T, in October 2017 for relapsed/refractory (R/R) large B-cell lymphoma after two or more prior lines of therapy based on results of the phase I Zuma-1 study[82,83]. Since then, axi-cel has been approved for second-line management of large B-cell lymphoma based on results from Zuma-9[84]. Other agents and indications have received approval - brexucabtagene autoleucel (brexu-cel) for R/R B-cell precursor acute lymphoblastic leukemia (ZUMA-3); tisagenlecleucel (tisa-cel) for R/R B-ALL (NCT02435849) and R/R large B-cell lymphoma (JULIET); idecabtagene vicleucel (ide-cel) for R/R multiple myeloma (KarMMa); abi-cel for R/R follicular lymphoma (FL) (ZUMA-5); and lisocabtagene maraleucel (liso-cel) for R/R large B-cell lymphomas (TRANSCEND). However, axi-cel's utility in solid tumors remains under investigation with modest results to date, partly because of tumor-suppressing molecules in the TME and tumor heterogeneity[85-91].

Several preclinical studies assessed potential targets for CAR-T in RCC, with CD70 as a promising site because it appears highly expressed in clear-cell and sarcomatoid tumors[92-94]. Early findings from the

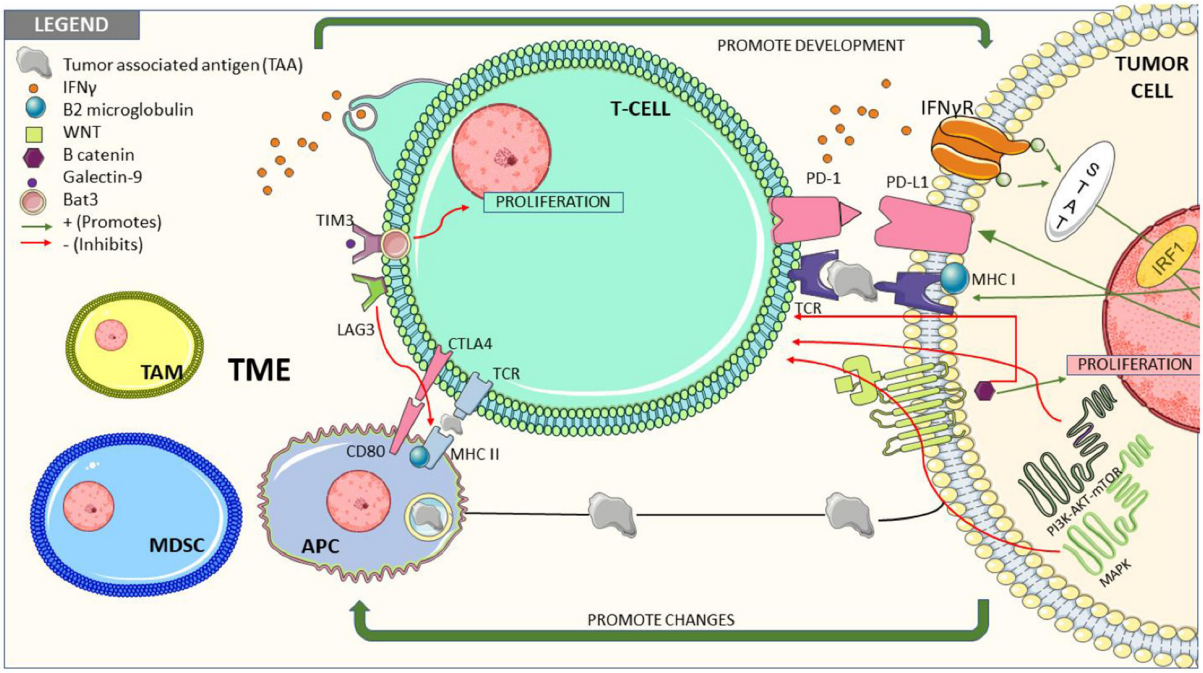

Figure 2. Tumor-intrinsic factors and the TME to describe potential mechanisms of resistance to immune therapies[61]. Reprinted with permission from Multidisciplinary Digital Publishing Institute. Abbreviation: TME, tumor microenvironment. Reproduced from[60] under the Creative Commons Agreement License.

COBALT-RCC trial (NCT04438083) were recently presented at the 2022 Society for Immunotherapy of Cancer Annual Meeting with safety and clinical activity for CTX130, an allogeneic anti-CD70 CAR-T that has been CRISP/Cas9 gene-edited, in the 13 evaluable patients so far. The ORR was 8% and the disease control rate 77%, with 1 partial response resulting in a CR maintained at 18 months[95]. TRAVERSE is an ongoing clinical trial studying the safety and efficacy of ALLO-360, an allogeneic CAR targeting CD70, in mRCC with preliminary data pending (NCT04696731). Another CAR-T product directed against carboxy-anhydrase-IX (CAIX) was promising in preclinical studies, though in an early phase trial, anti-CAR-T antibodies developed with immune responses, and no clinical benefit was noted, and the study was terminated[96]. This agent has again been evaluated now in combination with sunitinib, though in mouse models, with some synergistic response seen[97]. Ongoing studies are investigating other potential targets and methods of overcoming resistance and suppressive mechanisms of CAR-T by potentially combining it with other agents.

TIL therapy

TIL therapy has been investigated in mRCC for decades but with limited success, often because of suppressive features of the TME limiting retrieval of large amounts of tumor-reactive TILs[98-100]. Figlan *et al.* studied radical nephrectomy with IL-2 and TIL therapy among patients with mRCC with favorable results[101]. In a recent commentary, Andersen *et al.* highlighted several prior TIL studies in RCC with variable ORR ranging from 0% to 35%[99]. Ongoing studies for TIL therapy are limited though may benefit from combination-based strategies as previously considered, particularly with ICIs to help overcome TME-suppression coinhibitory signals[102]. Newer methods of TIL retrieval and expansion are also being explored to help overcome these limiting features[99,103]. Table 1 summarizes ongoing cellular therapy trials in mRCC.

Table 1. Summary of ongoing cellular therapy clinical trials in metastatic/advanced RCC. Information obtained from clinicaltrials.gov

Name	Identifier	Modality	Primary Site	Status
Clinical study of CAIX-targeted CAR-T Cells in the Treatment of Advanced Renal Cell Carcinoma	NCT04969354	CAR-T	Affiliated Hospital of Xuzhou Medical University	Recruiting (last updated June 2021)
Clinical study of CD-70 targeted CAR-T therapy in advanced renal cancer	NCT05420519	CAR-T	The Second People's Hospital of Shandong Province	Recruiting (updated June 2022)
Aldesleukin and pembrolizumab in treating patients with advanced or metastatic renal cell carcinoma	NCT03260504	CAR-T	Fred Hutch/ University of Washington Cancer Consortium	Recruiting (updated October 2022)
TIL Therapy for Metastatic Renal Cell Carcinoma	NCT02926053	TIL	Center for Cancer Immune Therapy, Denmark	Recruiting/ unknown (updated December 2019)
Safety and efficacy of ALLO-316 in subjects with advanced or metastatic clear-cell renal cell carcinoma (TRAVERSE)	NCT04696731	CAR-T	City of Hope, UCLA Medical Center, Moffitt Cancer Center, Memorial Sloan Kettering Cancer Center, Providence Portland Medical Center, MD Anderson Cancer Center	Recruiting (updated March 2022)
P-MUC1C-ALLO1 Allogeneic CAR-T cells in the treatment of subjects with advanced or metastatic solid tumors	NCT05239143	CAR-T	University of California San Francisco, Sarah Cannon Research Institute at HealthOne, University of Kansas Cancer Center, MD Anderson Cancer Center, NEXT Oncology	Recruiting (updated October 2022)
A clinical research about CD70-positive advanced/ metastatic solid tumors treated by CD70-targeted CAR-T	NCT05468190	CAR-T	Henan Cancer Hospital	Recruiting (updated September 2022)
A clinical study of CD70-targeted CAR-T in the treatment of CD70-positive advanced/ metastatic solid tumors	NCT05518253	CAR-T	First Affiliated Hospital, Zhejiang University	Recruiting (updated September 2022)
Safety and Efficacy of CCT301 CAR-T in adult subjects with recurrent or refractory stage IV renal cell carcinoma	NCT03393936	CAR-T	Shanghai Public Health Clinical Center	Active, not recruiting (updated October 2021)
A safety and efficacy study evaluating CTX130 in subjects with relapse or refractory renal cell carcinoma (COBALT-RCC)	NCT04438083	CAR-T	Multiple sites including the United States, Australia, Canada, and the Netherlands	Recruiting (updated May 2022)
HERV-E TCR Transduced Autologous T cells in People with Metastatic Clear-Cell RCC	NCT03354390	TCR	National Institutes of Health Clinical Center	Recruiting (updated December 2022)
Administering Peripheral Blood Lymphocytes Transduced with a CD70-Binding Chimeric Antigen Receptor to People with CD70 Expressing Cancers	NCT02830724	CAR-T	National Institutes of Health Clinical Center	Recruiting (updated January 2023)

Oncolytic virus-based therapy

Another avenue to overcome resistance is the combination of ICIs with oncolytic viruses, which elicit antitumor immunity[104]. Their use seems limited when used alone but may be more beneficial when combined with other agents targeting specific tumor-suppressive signals in the TME. In a phase II open-label study, patients with metastatic melanoma received either talimogene laherparepvec with ipilimumab or ipilimumab alone, and higher antitumor activity with a greater response rate was seen in the combination without increased toxicities (ORR 39%)[105]. Recently, a phase I study evaluating NeoVax, a neo-antigen cancer vaccine, with ipilimumab in RCC is actively recruiting (NCT02950766).

Other targeted approaches

As discussed previously, HIF-2a inhibitors are now being investigated in mRCC, and favorable outcomes led to the approval of belzutifan for VHL-associated tumors, including RCC. The role of HIF-2a inhibitors is being expanded and its use in combination with other agents, including ICIs, is being tested. Preclinical studies for belzutifan used in mouse models without HIF-2a expression found no efficacy for single-agent use, but there was potential synergy with checkpoint inhibitors[106].

Cyclin-dependent kinase 4/6 (CDK4/6) are signaling molecules that promote the progression of the cell cycle by overcoming the tumor suppressor activity of retinoblastoma. Inhibitors of CDK4/6 are currently FDA-approved in the management of breast and ovarian cancers with favorable response rates. Recent preclinical data show promise for CDK4/6 inhibition in RCC to potentiate response with ICIs and chemotherapy[107].

CONCLUSIONS

The management of mccRCC has progressed substantially with the advent of checkpoint inhibitors and targeted oral therapies, alone and/or in combination. However, innate or developed resistance to these therapies remains an ongoing challenge, particularly to ICIs. Several of the known mechanisms of resistance have been well defined, but recent progression in cellular therapies helps to expand the armamentarium of potential combination options that may overcome these modes of resistance and improve long-term disease control and survival for an otherwise dismal disease.

DECLARATIONS

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Author's contributions

Conceived and organized the design of the manuscript, performed the literature review and evaluation, and wrote the body of the manuscript: Chatwal MS

Provided administrative, technical, and material support and assisted in editing and reviewing the manuscript: Spiess PE

Performed the literature review, provided material and technical support, and helped to review and edit the manuscript: Chahoud J

Availability of data and materials

Not applicable.

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Conflicts of interest

Monica Chatwal has no conflicts of interest to report. Jad Chahoud reports advisory board consultation for Pfizer, Aveo, and Exelixis. Philippe Spiess has no conflicts of interest to report.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Commentary

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Major hurdles of immune-checkpoint inhibitors in pancreatic ductal adenocarcinoma

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Abstract

In 2030, pancreatic ductal adenocarcinoma (PDAC) will become the second leading cause of cancer-related mortality in the world. Unfortunately, neither conventional chemotherapy nor novel immunotherapeutic strategies can provide durable responses and the survival prognosis remains very low. PDAC is notorious for its immune-resistant features and unique genomic landscape facilitating tumor escape from immunosurveillance. Novel immune-checkpoint inhibitors (ICI) failed to show promising efficacy and other multi-modal approaches are currently being validated in multiple clinical trials. In this paper, we provide our opinion on the major mechanisms responsible for PDAC resistance to ICI therapy and provide our view on future strategies which may overcome those barriers.

Keywords: Pancreatic cancer, immune-checkpoint inhibitors, tumor resistance, microenvironment

Pancreatic ductal adenocarcinoma (PDAC) represents a major challenge in modern oncology[1]. It is predicted that by 2030 PDAC will become the second leading cause of cancer-related death[2]. Surgery is curative at earlier stages, whereas advanced or metastatic stages are almost impossible to treat[3].

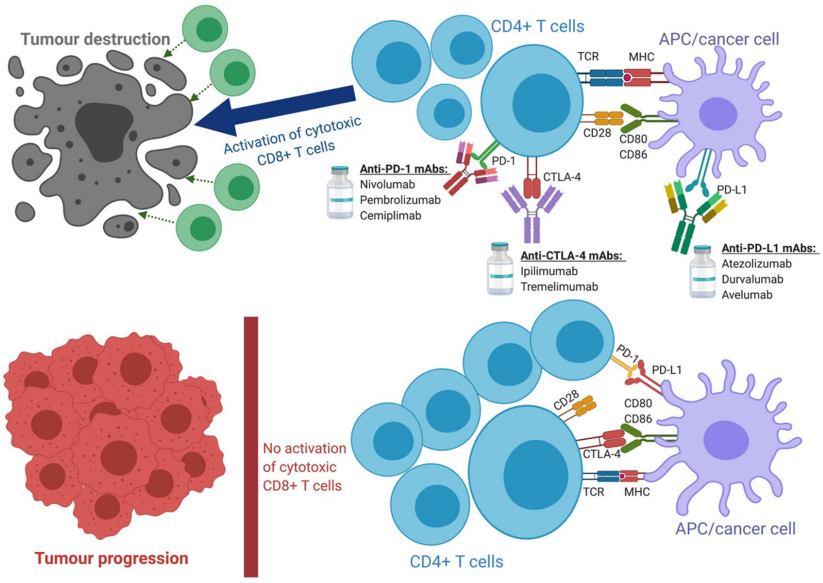

Figure 1. Mechanisms of immune-checkpoint inhibitors. ICIs target unique inhibitory checkpoint molecules expressed by T- and antigen-presenting cells. By blocking those receptors, ICIs promote the proper induction and differentiation of T cell-mediated immunity. In contrast, the absence of ICIs results in successful priming of checkpoint receptors with their ligands, thus inhibiting TCR activation overall, leading to cancer escape from immunosurveillance. APC: antigen-presenting cell; CD: cluster of differentiation; CTLA-4: cytotoxic T-lymphocyte associated antigen 4; mAb: monoclonal antibody; MHC: major histocompatibility complex; PD-1: programmed cell death protein 1; PD-L1: programmed cell death protein 1 ligand 1; TCR: T cell receptor.

Conventional chemotherapy can only provide a short partial remission with 5-year overall survival (OS) of less than 9% in patients with advanced PDAC[4]. Recent discoveries in cancer immunology have led to the successful use of immune-checkpoint inhibitors (ICIs) in treating advanced solid malignancies. ICIs are monoclonal antibodies that target immune checkpoints such as cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) with its ligands PD-L1/L2 and other expressed by antigen-presenting cells (APCs) and T cells [Figure 1][5]. ICIs have shifted treatment paradigms for melanoma, non-small cell lung cancer (NSCLC), and hepatocellular carcinoma[6,7]. Unfortunately, PDAC has shown incredible resistance to immunotherapy[8]. To date, US Food and Drug Administration (FDA) has only approved PD-1 inhibitor *pembrolizumab*, albeit only for patients with high microsatellite instability (MSI-H)[9]. Unfortunately, the majority of patients (~ 97%) with microsatellite stable status (MSS) are not benefited from ICIs and their outcomes remain critically poor[10]. Early trials combining chemotherapy with ICIs also fail to show any superior efficacy in MSS patients[9]. This paper provides an opinion on factors responsible for PDAC resistance to ICIs and potential strategies to overcome this issue.

Classically, PDAC has an immunologically "cold" tumor microenvironment[11] characterized by abundant infiltration of myeloid cells and a small number of infiltrating T- and NK (natural killer) cells [Figure 2]. A few studies suggested that focal adhesion kinases (FAK) can regulate the fibrotic features of cold tumors, including the immunosuppressive microenvironment[12,13]. The data from *in vitro* studies on the synergistic efficacy of FAK + PD-1 inhibitors showed promising responses and resulted in further testing of this regimen in clinical trials. Other factors of resistance are low mutational burden and complex immunosuppressive features able to inhibit T cell priming and trafficking, resulting in lower efficacy of immunotherapy[14].

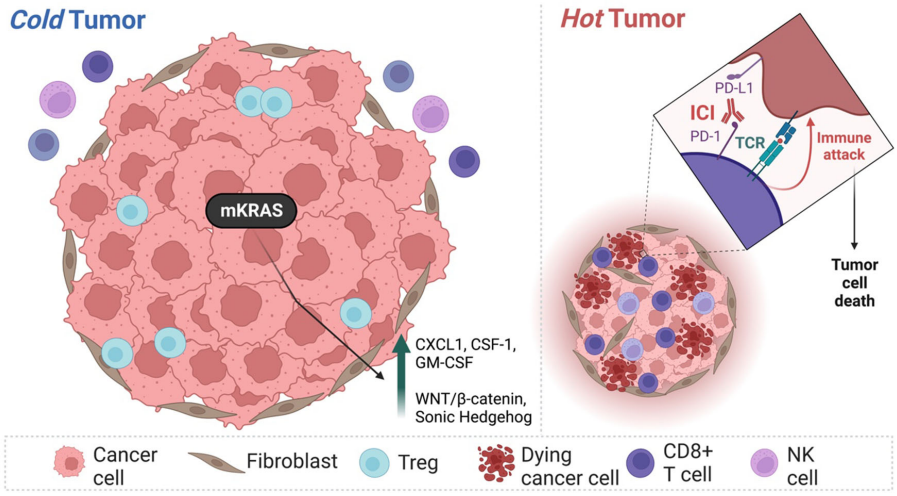

Figure 2. Mechanisms of pancreatic cancer resistance to immune-checkpoint inhibitor therapy. Pancreatic ductal adenocarcinoma is known as a tumor with a "cold" microenvironment characterized by a small number of CD8+ T- and NK cells, an abundance of regulatory T (immunosuppressive) cells, and poor response to ICI therapy. Mutation in *KRAS* gene (mKRAS) allows pancreatic cancer cells to induce expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine C-X-C motif ligand 1 (CXCL1) and C-C motif chemokine ligand 4 (CCL4) playing a crucial role in immunosuppression. Moreover, mKRAS leads to upregulation of WNT/β -catenin pathway and Sonic Hedgehog pro-inflammatory pathways overall, inhibiting the ICI therapy.

Stromnes *et al*. reported that analysis of tumor samples revealed that PDAC has a lower number of effector T cells and lower clonality of T cell receptors as compared to other solid tumors that can be successfully managed by ICIs[15]. Genome studies have established that PDAC almost ubiquitously has activating *KRAS* (Kirsten ras oncogene) mutations[16]. Conventionally, mKRAS is known to be associated with tumor proliferation and metastasis; however, recent results of high-throughput studies have established that mKRAS may orchestrate downstream signaling responsible for immunosuppression[17]. A few *in vitro* studies established that mKRAS inhibits the expression of MHC-I, CD47, and PD-L1[18,19]. It is known that PD-L1 is a crucial marker for ICI efficacy in non-small cell lung cancer[20]. Perhaps the lower expression of checkpoint proteins (targets) negatively impacts ICI therapy and explains its lower effectiveness in PDAC patients. Secondly, mKRAS can upregulate the expression of GM-CSF and CXCL1, which are involved in the recruitment of myeloid-derived suppressor cells known for their immunosuppressive features[21,22]. Furthermore, mKRAS can downregulate the expression of CCL4 via WNT/β -catenin pathway[23]. CCL4 is an important factor for recruiting dendritic cells; major APCs require FOR priming T cell response and activating the cytotoxic cascade[24,25]. A lower number of APCs impacts the tumor escape from immunosurveillance. Additionally, mKRAS promotes signaling via the Sonic Hedgehog pathway and can induce expression of matrix metalloproteinase 7 (MMP-7)[26] as well as selectively target lysosomal degradation of MHC-I molecules through an autophagy-dependent mechanism, thus negatively impacting ICI therapy[19]. Overall, it results in chronic inflammation and proliferation of the fibrotic stroma, thus complicating T cell trafficking[27]. The development of mKRAS-directed strategies may one day overcome this critical resistance mechanism and result in higher effectiveness of ICIs in PDAC.

In summary, PDAC is among the most immune-resistant tumors. Recent discoveries in understanding key elements of PDAC resistance to ICI therapy, including FAK[28], mKRAS and other novel molecules[29], have reshaped our view on future approaches for PDAC treatment. To effectively treat PDAC, it is crucial to elucidate the rational combinatorial approach(es) targeting both checkpoint proteins and non-redundant mechanisms of PDAC resistance, such as mKRAS. Moreover, novel therapeutic strategies should be selected based on patient's individual genotype, which is responsible for high phenotypic heterogeneity observed

across PDAC patients. Finally, mKRAS remains the bull's eye for PDAC immunologic resistance; thus, the combinatorial approach of ICI + MEK (mitogen-activated protein kinase) inhibitors should be thoroughly studied in randomized trials. The synergistic effect of both drugs may improve clinical outcomes for PDAC patients in the near future.

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Authors' contributions

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All authors equally contributed to this work.

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Cancer Drug Resistance

Review

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Importance of *ROS1* gene fusions in non-small cell lung cancer

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Abstract

Targeted therapy has become one of the standards of care for advanced lung cancer. More than 10 genetic aberrations have been discovered that are actionable and several tyrosine kinase inhibitors (TKIs) have been approved to target each of them. Among several genetic aberrations that are actionable in non-small cell lung cancer (NSCLC), *ROS1* translocations also known as gene fusion proteins, are found in only 1%-2% of the patient population. *ROS1* mutations can usually be detected using a combination of techniques such as immunohistochemistry (IHC), Fluorescence in-situ testing (FISH), polymerase chain reaction (PCR), and next-generation sequencing (NGS). However, RNA NGS and ctDNA NGS (liquid biopsies) also contribute to the diagnosis. There are currently numerous FDA-approved agents for these tumors, including crizotinib and entrectinib; however, there is in-vitro sensitivity data and clinical data documenting responses to ceritinib and lorlatinib. Clinical responses and survival rates with these agents are frequently among the best compared to other TKIs with genetic aberrations; however, intrinsic or extrinsic mechanisms of resistance may develop, necessitating research for alternative treatment modalities. To combat the mechanisms of resistance, novel agents such as repotrectenib, cabozantinib, talotrectinib, and others are being developed. In this article, we examine the literature pertaining to patients with *ROS1* tumors, including epidemiology, clinical outcomes, resistance mechanisms, and treatment options.

Keywords: *ROS1*, targeted therapy, non-small cell lung cancer

INTRODUCTION

Cancer is one of the leading causes of death in the United States. Over 1.8 million people are newly diagnosed with cancer every year, and 606,520 people lose their lives as a direct result of the disease. Three most common types of cancer are breast cancer, lung cancer, and prostate cancer[1]. There were 236,740 new cases of lung cancer, and over 130,180 people lost their lives to the disease[2]. Lung cancer is the most common cause of death resulting from any form of cancer, including breast, colorectal, prostate, and brain cancers.

Cancer of the lung is a disease that originates in the pulmonary parenchyma or the airways of the lungs. Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), which together account for 95% of all cases of lung cancer, are the two types of lung cancer that can be classified based on the histology. NSCLC includes adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, large cell carcinoma and sarcomatoid.

Previously, the treatment for lung cancer was only systemic chemotherapy that would target all cells that were proliferating or dividing which would prolong survival but would cause burden on quality of life of patients due to the toxic effects of treatment. Most recently, there has been a substantial amount of research on molecular pathways which allowed for target therapies to be developed to combat the pathways. Common molecular targets in lung cancer include EGFR, ALK, KRAS, ROS1, BRAF V600E, NTRK 1/2/3, METexon14, RET, ERBB2 and PD-L1. Molecular testing can be done with DNA sequencing, next generation sequencing (NGS), Fluorescence in-situ testing (FISH), immunohistochemistry (IHC) and liquid biopsies.

The availability of molecular testing, the identification of mutational drivers, and the introduction of targeted therapies have all changed the landscape of lung cancer therapy in recent years[1]. Epidermal Growth Factor Receptor (EGFR) mutations, for example, are known to contribute to the maintenance and functioning of cancer stem cells, including metabolism, immunomodulatory activity, dormancy, and therapeutic resistance[1]. While the *ALK* gene, located on the short arm of chromosome 2, is a transmembrane tyrosine kinase receptor which activates downstream signaling pathways resulting in uncontrolled cell proliferation and survival have been studied previously and are good therapeutic targets in the treatment of NSCLC[3,4]. The effectiveness of targeted therapy in treating EGFR[2-6] and ALK[7-11] mutations has increased interest in identifying additional oncogenic drivers with potential for clinical use.

The proto-oncogene *ROS1* on chromosome 6 encodes a tyrosine kinase receptor, limited to distinct epithelial cells during embryonic development[5] but with unknown functionality afterwards. It was originally identified in 1986 as the cellular homolog of the transforming *v-ros* sequence from the UR2 avian sarcoma virus[6] and later identified in glioblastoma-derived cells[7]. Further research has demonstrated ROS1's ability to cause cancer using in vitro and in vivo animal models[12]. The discovery of *ROS1* gene fusions in NSCLC has important clinical implications because it allows for the treatment of a subset of patients with a targeted therapy that can significantly improve their outcomes. It emphasizes the significance of extensive molecular profiling in NSCLC for identifying targetable mutations and guiding treatment decisions. Additionally, TKIs are effective on multiple targets including targeting the ALK rearrangement position, ROS1 fusions and NTRK rearrangements using agents such as ceritinib, crizotinib, lorlatinib and entrectinib[8].

Therapy options for NSCLC includes chemotherapy, immunotherapy and targeted therapy. Common chemotherapy options includes platinum agents (cisplatin, carboplatin) in combinations with taxanes (paclitaxel, albumin bound paclitaxel) or pemetrexed. Immunotherapy may be incorporated also using pembrolizumab, atezolizumab or even ipilimumab and nivolumab.

Molecular testing

Molecular testing is constantly evolving field and there is no single standard modality for detection of abnormalities. Different modalities of testing include DNA sequencing, NGS, FISH, IHC and liquid biopsies. DNA sequencing is the one of the oldest forms of testing for mutations which looks at an entire length of a single gene for mutation. The sensitivity is the lowest amongst all tests and may cause false negatives. The test requires the tumor cellularity to be high in the tissue sample to detect an abnormality. DNA and RNA NGS allows for testing of multiple genes at the same time or whole genomes with high sensitivity even if the tumor cellularity is low. The sensitivity is so high that it may detect even molecular alterations in the blood via circulating tumor DNA. FISH helps to examine gene rearrangements such as translocations, amplifications or deletions using DNA probes of various colors that move apart when gene has separated. IHC is both sensitive and specific. The turnaround time for results is rapid and it is the only test available to test for PD-L1 expression. Lastly, liquid biopsies allow for non-invasive and inexpensive means to test even when there is minimal tumor samples available for testing. It allows to monitor for disease response during treatment or even relapse in the future[9]. Liquid biopsies allow for detection of cell-free ctDNA in the blood of lung cancer patients. The negative aspects of the test is that there is a high false negative rate compared to standard tests due to small and variable amounts of circulating DNA that may be present. The sensitivity of liquid biopsies is 60% to 80% and sometimes cannot detect tumors that do not secrete DNA into the blood.

NSCLC GENOTYPES

ROS1 gene fusions

ROS1 gene fusions are genetic alterations that have been associated with the development and progression of multiple cancers, including NSCLC. The *ROS1* gene located on chromosome 6 (region 6q22.1) is responsible for the generation of two main splice variants of ROS1 that are encoded by either exon 43 or exon 44 and codes for a receptor tyrosine kinase that is essential for cell growth and differentiation. *ROS1* gene fusions occur when a portion of the gene joins with another gene, resulting in the production of a new, chimeric protein that promotes the growth of cancerous cells. *ROS1* fusion genes have been linked to a wide range of cancers ever since their discovery in the glioblastoma cell line U118MG in 1987[10-12]. *ROS1* is a proto-oncogene that encodes a receptor tyrosine kinase in humans, but its physiological function is not known. The somatic chromosomal fusions that involve ROS1 produce chimeric oncoproteins that are the driving force behind a wide variety of cancers, and a significant amount of interpatient partner-gene heterogeneity has been observed in various types of cancer. While ROS1 rearrangements are only present in approximately 1%-2% of patients with lung cancer, a significant number of people are affected by this mutation given high prevalence of lung cancer.

ROS1 fusions are most common in patients with NSCLC who are younger (median age of 50 years) and who have never smoked (80%)[12-15]. The CD74-ROS1 fusion is the most prevalent type of ROS1 fusion (44%), followed by the EZR-ROS1 fusion (16%), SDC4-ROS1 fusion (14%), and SLC34A2-ROS1 fusion (10%). Interchromosomal translocations are the major cause of recurrent ROS1 fusions in NSCLCs[12,15]. In model systems, ROS1 fusions on their own are sufficient to induce tumorigenesis; however, *ROS1* fusions working in conjunction with other aberrant oncogenes or tumor suppressor pathways can promote a significantly more aggressive form of disease. In some instances, *ROS1* fusions have been discovered to coincide with the presence of other oncogenic alterations. Other driver mutations, such as RET, NTRK, and

ALK fusions in NSCLCs, IMTs, and Spitzoid neoplasms, as well as FGFR2 and IDH alterations in cholangiocarcinoma[13,14], are likely to be mutually exclusive with the ROS1 fusions. In clinical practice, ROS1 fusion can be evaluated on a tissue biopsy and fluid cytology with ROS1 IHC. However, similar to the ALK IHC, the ROS1 IHC can report false positive results and thus, requires confirmation with NGS, ROS1 FISH, or with a multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) panel.

ROS1 gene fusions in NSCLC are typically detected via molecular testing, such as FISH or RT-PCR. These techniques enable the identification of the precise fusion partner and the development of therapies that inhibit the activity of the chimeric protein and plasma samples, the NGS can be used to analyze ROS1 rearrangement[13,14,16]. *ROS1* gene fusions in NSCLC have a significant potential therapeutic application. ROS1 inhibitors have shown remarkable efficacy in patients with ROS1-positive NSCLC, with response rates ranging from 60% to 80% and a median progression-free survival (PFS) of 12-19 months. This suggests that ROS1 inhibitors are highly effective. This is a significant improvement over conventional chemotherapy, which only has a moderate impact on NSCLC. Certain minority groups, including African-Americans, are disproportionately affected by lung cancer and may be more likely to have *ROS1* gene fusions. Understanding the epidemiology of ROS1 mutations in lung cancer, particularly in minority populations, is critical for improving cancer prevention, diagnosis, and treatment outcomes; similarly, understanding the prevalence, types, and characteristics of ROS1 fusion-positive mutations in NSCLC is critical for developing effective, patient-beneficial targeted therapies[13,16]. The use of appropriate diagnostic techniques to identify ROS1 fusion-positive patients, as well as the use of ROS1 inhibitors, has shown tremendous promise in the treatment of NSCLC.

EGFR mutations

Epidermal growth factor receptor (EGFR) mutations compromise 15% of NSCLC adenocarcinomas and occur often in non-smokers and Asian populations[17]. Common mutations in EGFR include exon 19 deletions or exon 21 L858R mutations which are very responsive to TKIs for treatment. Previously, first-generation TKI (gefitinib, erlotinib) and second-generation (afatinib) TKIs have been used for treatment but recently there has been improvement in survival with third-generation agents such as Osimertinib. Osimertinib is approved for first-line treatment in patients with EGFR exon 19 deletions or exon 21 L858R mutations. In the FLAURA study, a phase III trial, patients with treatment naïve EGFR-mutated NSCLC were randomized to receive Osimertinib versus the standard of care TKI (gefitnib or erlotinib). Osimertinib showed improvement in PFS 18.9 months versus 10.2 months, increased duration of response (DOR) (17.2 months versus 8.5 months) and overall survival (OS) 38.6 months versus 31.8 months[18]. Common adverse reactions include QTc prolongation and decreased left ventricular ejection fraction.

ALK mutations

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase that is often found to have chromosomal rearrangements involving *ALK* gene on chromosome 2 and compromise 3%-5% of NSCLCs[19,20]. The rearrangement involves the 5' end of the echinoderm microtubule-associated protein-like (*EML4*) gene with the 3' end of the *ALK* gene, creating a fusion oncogene *EML4-ALK*[21]. ALK mutations are often found in never or light smokers and younger populations. Common ALK inhibitors include alectinib, brigatinib, certinib, lorlatinib and crizotinib. In the ALEX study, 303 patients were randomly selected to receive alectinib versus crizotinib. Alectinib showed a decreased risk of progression or death in 53% of patients, PFS was 35 months versus 11 months in the crizotinib arm and OS was not reached[22]. Common side effects included anemia, myalgia, hyperbilirubinemia, weight gain and photosensitivity.

MET mutations

MET is a tyrosine kinase receptor for hepatocyte growth factors. MET mutations include MET exon-14 skipping mutations (3% of NSCLC) and *MET* gene amplification (2%-4% of NSCLC). MET exon-14 skipping mutations decreases the degradation of MET which causes it to become a oncogenic driver. Capmatinib, crizotinib and tepotinib have been both approved for treatment of MET exon-14 skipping mutations. In the GEOMETRY-mono-1 trial included 97 patients with MET exon-14 skipping mutations and capmatinib showed a 68 percent overall response rate and the PFS was 12.4 months. Common adverse reactions included peripheral edema, nausea, vomiting and increased creatinine[23]. Lastly, in *MET* gene amplifications capmatinib or crizotinib is often used.

RET rearrangements

The rearranged during transfection gene (*RET*) translates a cell surface tyrosine kinase receptor kinase that is mutated. RET rearrangements are present in 1%-2% of NSCLC, often in non-smokers and younger patients. First line therapy include treatment with selpercatinib, pralsetinib and cabozantinib. Common side effects include hypertension, fatigue, diarrhea, transaminitis and pneumonia[24-26].

BRAF mutations

BRAF is involved with downstream signaling of Kristen rat sarcoma viral oncogene homolog (KRAS) and activates the mitogen-activated protein kinase (MAPK) pathway. BRAF mutations are present in 1%-3% of NSCLC and are present in patients with a smoking history. BRAF mutations can occur in the V600 position of exon 15 or outside of the domain. First line therapy options include dabrafenib, dabrafenib/trametinib and vemurafenib.

NTRK fusions

NTRK fusions are very rare (< 1% of NSCLC) and involve one of three tropomyosin receptor kinases (TRK). Therapy options for NTRK fusions include Larotrectinib and Entrectinib. Larotrectinib was analyzed in several phase I/II trials which showed an ORR 79%. Most common adverse effects included elevated transaminases, anemia and neutropenia[27]. Entrectinib showed a ORR 57% in several trials[28]. Entrectinib and Larotrectinib have not been compared with each other.

KRAS mutations

The Kristen rat sarcoma viral oncogene homolog (KRAS) occurs in 20% to 25% of NSCLC and is often associated with smoking[29]. In NSCLC, the G12C mutation is targeted by sotorasib and adagrasib as subsequent therapy after patients have received 1 prior therapy. The most common adverse reactions include elevated transaminases and diarrhea.

Mechanisms of resistance

As was mentioned before, systemic or central nervous system resistance often develops after good clinical responses and survival. Mechanisms of resistance to ROS1 can be categorized as on-target or off-target. After a tumor has been treated with a TKI, on-target resistance mechanisms can emerge, such as ROS1 mutations[30-32]. There are several mutations described, the most commonly recognized of which is G2032R, followed by D2033N, and both of them are solvent-front mutations. Other acquired resistance mutations included gate keeper mutations and among them S1986F/Y, L2000V, F2004V, L2026M, and G2032K[33-36]. L2086F is an important and very dangerous resistance mutation because it is resistant to all TKIs (crizotinib, lorlatinib, taletrectinib) [Figure 1][30,31,34].

TKIs have varying efficacy against resistant mutations, sensitivity must be confirmed prior to initiating therapy. For example, lorlatinib has efficacy against the K1991E or S1986F resistant mutations, but has

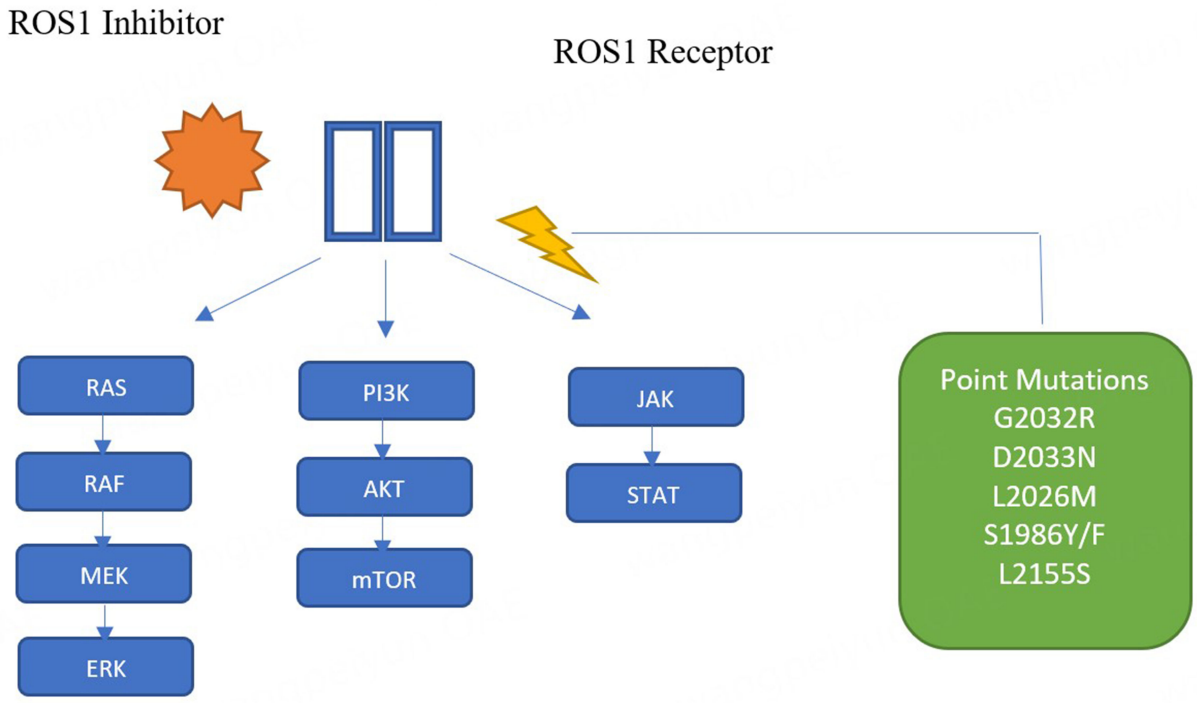

Figure 1. ROS1 pathway and resistance mechanism.Molecular mechanisms of ROS1 Inhibitors in ROS-1 rearranged lung cancer patients. Molecular pathways to resistance include an activated ROS1 kinase which activates the SHP-2 phosphatase and increases the JAK/STAT3, PI3K/AKT/mTOR, RAS/RA/MEK/ERK signaling pathways to promote growth and survival of the cell.

limited therapeutic potential against the G2032R mutation[35,36], and may be used after failure on entrectinib[37]. Sequential use of crizotinib and lorlatinib has led to the formation of a compound mutation of G2032R/L2086F but cabozantinib has potential to overcome this compound mutation[30,31,34]. Cabozantinib is frequently used to treat thyroid cancer as well as other tumors that it can selectively target, including MET, VEGFR-2, RET, ROS-1, and AXL, with good penetration of blood brain barrier. Despite the presence of resistance mutations such as D2033N or G2032R[38-40], resistance to crizotinib, ceritinib, and entrectinib can be overcome with cabozantinib. Brigatinib, an additional ROS1 inhibitor, has demonstrated antitumor activity against a number of crizotinib-resistance mechanisms[41], including the L2026M mutation, but not the G2032R mutation[41-43]. Taletrectinib, ROS1 and NTRK inhibitor with activity against G2032R, L1951R, S1986F and L2026M mutations, but minimal activity against D2033N mutation. A clinical trial with 46 patients on previous multiple lines of therapy had a 33% ORR and another trial with 15 patients, reported 58.3% ORR in previously exposed patients and 66.7% in treatment naïve patients[30,44]. There have been additional intrinsic mutations discovered in addition to the ones described and shown in Table 1.

Additionally, there are important off-target resistance mechanisms that involve the presence of other genomic aberrations like: *MET* amplification (3%), *KRAS* mutations (20%-25%) or small cell transformation (3%-10%)[45-47]. Lastly, the molecular pathways to resistance includes an activated ROS1 kinase activates the SHP-2 phosphatase and increases the JAK/STAT3, PI3K/AKT/mTOR, RAS and MAP/ERK signaling pathways to promote growth and survival of the cell[48] and HER2 mediated bypass signaling [Figure 1].

ROS1 TARGETED THERAPIES

Numerous tyrosine kinase inhibitors (TKIs) have been developed, such as crizotinib, ceritinib, entrectinib,

Table 1. Intrinsic mutations in ROS1 resulting in resistance

Intrinsic	Crizotinib	Ceritinib	Lorlatinib	Brigatinib	Cabozantinib	Foretinib	Entrectinib	Repotrectinib	Ensartinib
G2032 R/K	R	R	R	R	S	S	R	S	R
D2033	R	R	S	R	S	S	R	S	R
L1951	R	R		R	S				
L2026	R	R	S	R	S	S	R		
L1196M	R								
S1986 Y/F	R	R	S						
L2086F	R								
E1935G	R	R				S			
L1947R	R					S			
G1971E	R					S			
L1982F	R				S	S			
C2060G	R					S			
V2098I	R	S		S	S	S			
L2155S	R	R							
E1990G	R	R			S				
F1994L	R	R			S				
F2004 C/I	R	R	S	S	R	S			

Blank: No available data; R: resistant; S: sensitive.

and lorlatinib. Both crizotinib and entrectinib have been approved for use in the first-line setting. According to Table 2, the use of a ROS1 TKI in the first-line setting improves OS, response rate (RR), and progression-free survival (PFS) in comparison to standard chemotherapy[24]. Table 3 depicts the most prevalent adverse events associated with each targeted therapy.

Crizotinib

Crizotinib was the first TKI to be approved for management of ROS1 mutated NSCLC. In 2014, the PROFILE 1001 study showed significant activity against ROS1-rearranged NSCLC[49]. PROFILE 1001 was a multicenter, single-arm phase I study that included patients with metastatic ROS1 positive NSCLC that depicted an ORR 72%, PFS 9.2 months and median DOR 17.6 months[49]. An updated analysis of the PROFILE 1001 in 2019, median OS was 51.4 months, median PFS 19.3 months and median DOR 24.7 months[50].

In a prospective phase II OxOnc study, crizotinib demonstrated an ORR 91%, DOR 19.7 months, PFS 15.9 months and OS 32.5 months[51]. In comparison to the PROFILE 1001 study, the OxOnc study included patients with brain metastases (BM) both symptomatic and asymptomatic comprising 18% of study population. The study showed a PFS of 10.2 months (patients with BM) *vs.* 18.8 months (patients without BM)[51]. Additionally, another phase II multicenter study, evaluated not only ROS1 rearrangement but also co-mutations with TP53. The results of the phase II study showed ORR 70% with median PFS 20 months, while those with TP53 co-mutations had a shorter median of PFS of 7 months[52].

Failure to respond to crizotinib frequently results from CNS progression and mutations. G2032R/K, D2033N, S1986Y/F, L2026M, and L1951R are common mutations that cause crizotinib to fail, with G2032R being the most common[53]. Lorlatinib is a potential agent that can be used in patients that have progression on crizotinib as it is able to overcome resistance mutations.

Table 2. Clinical activity and toxicity profiles of ROS1 tyrosine kinase inhibitors in treatment naïve patients

TKI	RR (%)	PFS (months)	OS (months)/(1 yr)
Crizotinib[49,50]	72	19.3	51.4/79
Ceritinib[56]	67	19.3	24/56
Entrectinib[62]	53	19	NR/85
Lorlatinib[64]	21	21	NR

OS: Overall survival; PFS: progressive free survival; RR: response rate; TKI: Tyrosine Kinase Inhibitor.

Table 3. Summary of adverse events from ROS1 tyrosine kinase inhibitors

Tyrosine Kinase Inhibitor	Common TRAE	Grade 3-4 TRAE (%)
Crizotinib[49,50]	Visual impairment, diarrhea, constipation, peripheral edema, nausea, elevated AST, dizziness	36
Ceritinib[56]	Diarrhea, nausea, anorexia, vomiting, cough, elevated creatinine, elevated transaminases	37
Entrectinib[62]	Dysgeusia, fatigue, dizziness, constipation, nausea, weight gain, paresthesia	34
Lorlatinib[64]	Hypercholesterolemia, hypertriglycerdiemia, edema, peripheral neuropathy, AMS, weight gain, dizziness	49

AMS: Altered mental status; AST: aspartate aminotransferase; TRAE: treatment related adverse events.

In a phase I/II trial with ROS1-positive patients who have received crizotinib previously led to an ORR of 35%[54]. Additionally, cabozantinib is a potential agent that be used to overcome crizotinib resistance[38]. Crizotinib also has very poor blood-brain barrier penetration and is often limited for use in patients with CNS progression[53]. Lastly, common side effects of crizotinib include vision disorder, diarrhea, nausea, vomiting and peripheral edema[49].

Ceritinib

Ceritinib is a second-generation TKI approved to treat NSCLC patients with ROS1 rearrangement. Ceritinib is a highly potent TKI with a potency 20 greater than crizotinib[55]. Ceritinib efficacy was evaluated in a multicenter, phase II study of 32 patients with ROS1 rearrangement and the results showed a ORR of 67%, median PFS 19.3 months and median OS 24 months[56]. Most common side effects included diarrhea, nausea and anorexia[56]. In 2017, the ASCEND-8 trial evaluated if a decreased dosage of ceritinib had similar efficacy with less adverse risk. Certinib 450 mg was compared to the standard 750 mg dosage and similar efficacy but more tolerable side effects were seen using the lower dosage[57]. Although ceritinib is a highly effective TKI in the treatment of ROS1-mutated NSCLC, it has limited applications in crizotinib-resistant patients because it is resistant to the common mutations seen with crizotinib, such as G2032R, D2033N, L1951R, and S1986Y/F[55].

Entrectinib

Entrectinib is a ROS1 inhibitor with profound penetration in the CNS allowing it to exert its anti-tumor activity[58]. In the STARTRK-1, STARTRK-2 and ALKA-372-001 trials, 53 ROS1 mutated treatment naïve patients were given entrectinib 600 mg daily and followed for at least 12 months. The patients were evaluated for ORR and DOR as co-primary endpoints while OS, PFS, intracranial DOR (IC-DOR), intracranial ORR (IC-ORR) and safety effect profile were secondary endpoints. In the studies, most patients were white (59%), female (64%) and never smokers (59%) with baseline CNS disease present in over 43% of patients[59-61]. Forty-one patients were found to have a response, 6% of patients had a complete response (CR), 72% had a partial response (PR) and 2% had an objective response (OR). The median DOR was 24.6 months, ORR 77% and median PFS 19 months in patients without CNS disease. Median OS was not met at 15.5 months during follow-up. While on the contrary, patients with CNS disease had a median DOR 12.6

months, ORR 74%, median PFS 13.6 months, IC-DOR 12.9 months and IC-ORR was 55%. The study showed that entrectinib was not only active systemically but also had good CNS penetration. The most common side effects included nervous system disorders (3%) and cardiac disorders (2%)[62].

Lorlatinib

Lorlatinib is a selective third-generation ALK and ROS1 TKI that has both systemic and CNS activity via reduction of P-glycoprotein 1-mediated efflux[63]. In a phase I study, Lorlatinib was given to ROS1-positive NSCLC patients with ECOG status 0-1. The primary endpoint was dose-limiting toxicities and the secondary endpoints were pharmacokinetics, safety, and overall response. Lorlatinib has good penetration in the CNS with a response of 60%. Common adverse effects were weight gain, peripheral edema and constipation.

In a phase II trial, 69 patients were enrolled with ROS-1 positive NSCLC with 30% being TKI naïve and 70% having previous TKI exposure (58% were pretreated with crizotinib and 12% with other TKIs) to evaluate for an overall and intracranial response. The RR was 62% in the TKI naïve patients and 35% in the TKI exposed patients, while the PFS was 21 months *vs.* 8.5 months. Intracranial responses were seen in 64% of TKI naïve and 50% in those pre-treated with crizotinib. The most common side effects included hypertriglyceridemia and hypercholesteremia[64].

In the PFROST study, predictive molecular events for response were evaluated. All patients in study were ROS1 mutated and had been previously treated with crizotinib. Prior to initiation of therapy with lorlatinib, the patients had a tissue or blood sample taken. Patients with a G2032R mutation progressed rapidly and continued to have this mutation at the time of treatment failure, showing the importance of early testing to predict response to therapy[35].

Lastly, the efficacy of lorlatinib was tested in the French LORLATU and Asian GLASS studies, which revealed significant RR both extracranial and intracranial, confirming lorlatinib's potency as a good therapeutic option in the treatment of ROS1 mutated NSCLC[65,66].

Novel agents

First-generation TKIs are effective in treating ROS1+ NSCLC until resistance mutations develop, presenting a clinical challenge. Chemotherapy is still used as a last resort after disease progression occurs as a result of resistance mutations. Chemotherapy is not as effective as TKIs, with a median PFS of 7 months[44]. Studies for potential newer second-line regimens are ongoing.

Cabozantinib

Cabozantinib is a tyrosine kinase inhibitor that is currently approved for treatment of renal cell carcinoma, hepatocellular carcinoma and medullary thyroid cancer[39]. In a recent study, four patients were evaluated that developed resistance to first line TKIs, crizotinib and ceritinib, and were subsequently treated with cabozantinib. In the study, an OR 25 percent and PFS from 4.9 to 13.8 months was achieved. Study limitations included no pre-therapy tissue samples to identify resistance mutations and no clear understanding whether the effects were due to ROS1 inhibition or direct drug effects[67]. Cabozantinib is very effective against most mutations including G2032R and D2033N but has significant toxicity that limits research on the medication for ROS1+ patients[58].

Taletrectinib (DS-6051b)

Taletrectinib is a TKI that has activity against both NTRK and ROS1. Recently, it was found that Taletrectinib can be used in ROS1 positive patients that harbor CD74, S1986F, L2026M, L1951R and

G2032R mutations[39]. In a phase I study, 15 Japanese patients were enrolled with 12 having measurable lesions and 9 were treatment naïve. In treatment naïve patients, the ORR was 66.7 percent, while those that were pre-treated with TKIs the ORR was 33.3 percent[44]. Most recently, two phase I studies in the United States and Japan evaluated an ORR, PFS and safety. Twenty-two patients with ROS1 positive NSCLC were evaluated and given Taletrectinib in dose escalation. The ORR for ROS1 TKI naïve patients was 66.7 percent, while for previously treated patients it was 33.3 percent. The PFS for TKI naïve patients was 29.1 months, in contrary to pre-treated TKI patients it was 14.2 months[68]. Taletrectinib was found to have good systemic activity in patients with ROS1 positive NSCLC regardless of TKI pretreatment. Most common side effects include elevated transaminases (27%) and GI toxicity (4.5%).

Repotrectinib (TPX-0005)

Repotrectinib is a selective tyrosine kinase inhibitor that not only inhibits NTRK and ALK but also ROS1 with a potency level of > 90 folds of crizotinib and is able to overcome G2032R and D2033N mutations[69]. Repotrectinib has both systemic and CNS activity[70]. Recently, in the TRIDENT-1 trial, 33 ROS1 positive patients were evaluated that included both TKI naïve and pre-treated TKI patients. The patients received dose escalations of repotrectinib with a ORR 82 percent (regardless of dose) in TKI naïve patients and 39 percent in pre-treated TKI patients. The ORR increased to 55 percent in pre-treated TKI patients with dosages of 160 mg. The intracranial-ORR was 100 percent in treatment naïve patients and 75 percent in those previously treated with TKI. The median DOR was 23.1 months, while the PFS was 24.6 months[71]. The study demonstrates that repotrectinib is a potent TKI that can be used in both treatment naive and previously treated TKI patients with ROS1 positivity. More research is required, and it is currently underway in phase II of the Trident study, which is scheduled to conclude in 2023. The TRIDENT study's Phase II is enrolling 190 ROS1 positive patients and evaluating response based on the number of TKIs previously used. Dizziness, fatigue, constipation, and dyspnea are the most common side effects.

CONCLUSIONS

ROS1 fusions are not very common and only represent around 2% of NSCLC, however due to the high incidence of lung cancer in the US, this genetic aberration is present in several thousand patients.

Thanks to DNA NGS and other diagnostic methods in tissue or blood we are able to detect these genetic aberrations and offer our patient's front-line therapy with very good clinical outcomes, significant survival prolongation and good quality of life.

Currently, crizotinib and entrectinib are approved for ROS1 tumors, however ceritinib and lolartinib have already showed good clinical efficacy too. Repotrectinib, talotrectinib and cabzantinib are in development to fight on-target mechanisms of resistance as future therapeutics in the combat against ROS1 fusions.

DECLARATIONS

Authors' contributions

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Cancer Drug Resistance

Review

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The evolving role of DNA damage response in overcoming therapeutic resistance in ovarian cancer

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Abstract

Epithelial ovarian cancer (EOC) is treated in the first-line setting with combined platinum and taxane chemotherapy, often followed by a maintenance poly (ADP-ribose) polymerase inhibitor (PARPi). Responses to first-line treatment are frequent. For many patients, however, responses are suboptimal or short-lived. Over the last several years, multiple new classes of agents targeting DNA damage response (DDR) mechanisms have advanced through clinical development. In this review, we explore the preclinical rationale for the use of ATR inhibitors, CHK1 inhibitors, and WEE1 inhibitors, emphasizing their application to chemotherapy-resistant and PARPi-resistant ovarian cancer. We also present an overview of the clinical development of the leading drugs in each of these classes, emphasizing the rationale for monotherapy and combination therapy approaches.

Keywords: Ovarian cancer, platinum resistance, PARPi resistance, DDR

OVARIAN CANCER AND PLATINUM-BASED CHEMOTHERAPY

Ovarian cancer is the deadliest gynecologic malignancy, estimated to account for 12,810 deaths in 2022[1]. Epithelial ovarian cancer (EOC) is treated in the first-line setting with combined platinum and taxane chemotherapy[2]. Over 80% of high-grade serous ovarian cancers (HGSOC), the most common ovarian cancer subtype, will exhibit an initial response to platinum-based chemotherapy[3]. Defects in homologous recombination repair (HRR) are present in about 50% of EOC, and the initial sensitivity of EOC to platinum-based therapy has been attributed to the high prevalence of homologous recombination deficiency (HRD) in this cancer type[4]. Only a minority (10%-15%) of HGSOC will not demonstrate a meaningful initial response to first-line platinum-based chemotherapy[3]. Regardless of the initial response to platinum-based chemotherapy, the majority of patients with EOC will go on to develop disease recurrence. Most recurrent EOC will eventually exhibit platinum resistance following treatment with one or more lines of chemotherapy[2]. In this regard, ovarian cancer exhibits both intrinsic and acquired resistance to platinum-based chemotherapy.

Multiple mechanisms of inherent and acquired resistance to platinum-based chemotherapy have been described. One of these is the alteration of volume-regulated anion channels (VRACs) which regulate the influx of platinum compounds into cells[5,6]. Additional mechanisms of platinum resistance include altered intracellular sequestration of platinum, changes to the tumor microenvironment, and altered recognition and repair of DNA damage[4,5]. Alterations in the DNA damage response (DDR) are being studied as a potential vulnerability that can be exploited for the treatment of EOC. It has been demonstrated that acquired alterations in key DDR genes following PARPi therapy, like reversion mutations in *BRCA*, *RAD51C*, and *RAD51D,* resulting in restoration of homologous recombination, are associated with the development of post-treatment resistance[7]. In this review, we will focus on the role of DDR in the current landscape of treatment for recurrent EOC.

PARP INHIBITION IN OVARIAN CANCER

PARP inhibitors were the first drug class to exploit synthetic lethality for the treatment of ovarian cancer[8]. Synthetic lethality is the concept whereby an inactivating mutation in one gene (or inhibition of its protein product) is innocuous, but inactivating mutations in two genes (and/or inhibition of their protein products) results in cell death[8]. The proteins encoded by the *BRCA1* and *BRCA2* genes are critical for homologous recombination, a DNA damage repair pathway for double-strand break repair. The PARP1 protein is involved in single-strand break repair via base excision repair[9]. A PARPi is lethal only to cells with a predisposing defect in homologous recombination[8]. PARPi therapy has been most effective in patients with germline *BRCA1* or *BRCA2* mutations, whose tumors likely exhibit deficient homologous recombination. Maintenance therapy with the PARPi olaparib after first-line chemotherapy in patients with *BRCA*-mutated advanced ovarian cancer showed dramatic improvements in progression-free survival (PFS) compared to placebo[10]. Patients with germline *BRCA1* or *BRCA2* mutations account for about only 15% of all patients with ovarian cancer, but approximately 50% of epithelial ovarian cancers harbor defects in HRR[11,12]. PARP inhibitors have shown some activity in patients who do not carry *BRCA* mutations but whose tumors exhibit HRD based on the result of a somatic profiling assay (ex. Myriad MyChoice CDx HRD)[10,13,14]. On April 29, 2020, the FDA approved niraparib as first-line maintenance for all advanced EOC following first-line platinum-based chemotherapy, regardless of *BRCA* status or HRD status. The EMA made a similar approval on October 29, 2020.

The majority of patients treated with PARP inhibitors in the first-line setting will go on to experience disease recurrence[10,14]. Multiple mechanisms of PARPi resistance have been described. These include reversion mutations in *BRCA1* or *BRCA2*, which restore HRD[15], downregulation of non-homologous end-

joining (NHEJ), loss of 53BP1[16], and enhanced replication fork protection[17,18]. Cells that exhibit HRD, such as *BRCA* deficient cells, can also defer to a more error-prone polymerase theta-mediated end-joining (TMEJ, a.k.a. alt-NHEJ or microhomology-mediated end-joining, MMEJ) as a backup pathway to repair double-strand breaks. TMEJ-mediated repair involves PARP1, DNA ligase III, and DNA polymerase theta (Polθ)[19].

Polθ is encoded by the gene *POLQ*. Polθ has become a therapeutic target of interest in cancers due to evidence of synthetic lethality when there is a loss of *POLQ* and dysregulation or loss of other DNA repair-related tumor suppressor genes that control double-strand break repair or HRR[20]. Some recent studies suggest that secondary mutations restoring BRCA1/2 function are caused by the activity of TMEJ with Polθ facilitation. Ceccaldi *et al.* provided *in vitro* evidence that HRR-deficient ovarian cancer cells were dependent on Polθ[21]. ART558, a small molecule inhibitor of Polθ, elicited DNA damage in *BRCA1*- and *BRCA2*- mutant tumor cells and was shown to enhance the effects of a PARPi in *in vitro* and *in vivo* models[22]. ART 558 is expected to move forward in Phase I trials. It has also been proposed that cancer stem cells (CSCs) might display an inherent or acquired resistance to PARPi. Using *in vitro* and *in vivo* preclinical models of ovarian cancer, it was shown that PARP inhibition primarily targeted the non-cancer stem cell populations. While the CSCs showed increased evidence of DNA damage in response to PARP inhibition, the CSCs were able to repair their damaged DNA more efficiently than their non cancer stem cell populations[23]. DMC1, a meiotic-specific recombinase, which has been shown to be expressed in cancers, was proposed as a possible mediator of this purported resistance. Interestingly, recent research shows that diverse mechanisms of resistance can emerge within a *BRCA1* mutant cell line, and multiple mechanisms of resistance may emerge within even a single clone. For example, a single clone has been shown to demonstrate restoration of RAD51 foci formation and decreased levels of PARylation, suggesting downregulation of PARP1[17]. Given the multiple described mechanisms of acquired resistance to targeted small molecule inhibitors, overcoming therapeutic resistance is an ongoing challenge to investigators and clinicians alike[24,25]. Other combination strategies designed to target the CSCs as well as the more differentiated tumors cells have been investigated in preclinical ovarian cancer models, including those targeting the metabolic pathway, aldehyde dehydrogenase activity, and long non-coding RNAs in combination with either a cytotoxic or another biologic agent[26-28]. Others have shown that metformin could reduce CSC populations and increase sensitivity to cisplatin[29]. The diversity in the CSC populations from patient to patient is likely to have some impact on their success in the clinic.

TARGETING DDR PROTEINS IN THE PARPI-RESISTANT OR PLATINUM-RESISTANT SETTING

The current treatment paradigm for PARPi-resistant and platinum-resistant HGSOC does not address specific mechanisms of resistance. Instead, treatment typically shifts away from therapies dependent on deficient HRR[30]. To this effect, PARP inhibitors have shown very poor efficacy in the heavily pretreated setting[31]. These findings suggest the need for an alternative approach to treatment for EOC that is resistant to both platinum-based chemotherapy and PARP inhibitors.

It is well understood that the cancer genome is less stable than that of healthy cells[32], and tumor cells demonstrate high rates of DNA replication and division. HGSOC has a high incidence of inactivating *TP53* mutations[33]. *TP53* encodes p53, a critical tumor suppressor that protects cells from proceeding through the cell cycle in the setting of DNA damage[34]. The cooccurrence of inactivating *TP53* mutations and mutations in *BRCA1* and *BRCA2* has been suggested to be a critical element in the development of *BRCA* null tumors[35,36]. As such, PARPi resistance almost always occurs in the setting of deficient p53. In the setting of DNA damage, p53-deficient cells will be preferentially dependent on alternative cell cycle checkpoints. This

selective dependence is being explored as a potential target for EOC treatment[37,38].

While many mechanisms are likely at play, cancer cells ultimately attempt to replicate damaged DNA, leading to "replicative stress". Genomic instability, resulting from an accumulation of DNA damage, is a hallmark of many cancers, including ovarian cancer. Accumulation of DNA damage can result in replication stress. ATM and ATR are kinases that the cells rely on to alleviate replication stress. Specifically, ATM/ATR signaling arrests the cell cycle via phosphorylation of downstream kinases such as CHK2 and CHK1, respectively. If ATM/ATR fails, the replication fork becomes unstable and can collapse[39,40]. To this end, these proteins critical to DDR (ATR, ATM, CHK1, CHK2) are being studied as potential therapeutic targets. This strategy relies on the hypothesis that inhibition of various DDR pathways may confer a therapeutic effect in heavily pretreated EOC that is resistant to either platinum-based chemotherapy, PARP inhibitors, or both. Inhibitors of ATR, ATM, CHK1, CHK2, and WEE1 are in clinical trials. Here we discuss inhibitors of ATR, CHK1, and WEE1 in more detail, as they have progressed the furthest in clinical development.

ATR

The serine/threonine kinase ATR functions as one of the cell's master regulators of genotoxic stress. Some forms of DNA damage can result in tracts of single-stranded DNA. In addition, segments of single-stranded DNA may be formed at stalled replication forks when the activities of the replication helicase and DNA polymerase are uncoupled[41]. Replication protein A (RPA) is recruited to regions of single-stranded DNA (ssDNA). ATR then localizes to regions of single-stranded DNA (ssDNA) via its binding partner ATRIP (ATR interacting protein), a process dependent on RPA [Figure 1][42]. Following ATR localization to RPA, multiple activator proteins, including TopBP1 and ETAA1, are required for ATR activation[41]. When active, ATR phosphorylates its immediate downstream effector CHK1. Moreover, ATR plays a key role in preventing replication fork collapse by limiting CDK (cyclin-dependent kinase) signaling, which restrains replication fork firing[43]. ATR also directly targets helicases, preventing unstable replication fork configurations, and ATR regulates deoxyribonucleotide availability in response to DNA damage. It has been proposed that replications forks may be more prone to collapse without these interventions[41].

The high burden of replication stress in cancer cells suggests that malignant cells may be especially sensitive to ATR inhibition. To this end, ATR inhibitors have proven effective in slowing the proliferation of *BRCA2*-mutant HGSOC cell lines and tumors[44], with synergistic effects following co-treatment with platinum-based chemotherapy or PARP inhibition. Using organoid models of HGSOC, recent studies have leveraged the ability to monitor homologous recombination defects to predict sensitivity to ATR inhibition[45,46].

ATR inhibition has been studied in ovarian cancer as a mechanism for overcoming PARPi resistance[47]. Supporting this hypothesis, PARPi-resistant, BRCA1-null cells have been shown to increase dependence on ATR[48]. Two trials investigating combined ATR and PARP inhibition among patients with PARPi-resistant recurrent ovarian cancer are ongoing. CAPRI is a phase II clinical trial of olaparib in combination with the ATR inhibitor ceralasertib (AZD6738) in patients with recurrent ovarian cancer; data on the platinum-resistant ovarian cancer cohort of this trial has been published[49]. Among the 12 PARPi-naïve patients who were evaluated, the best response was stable disease in nine patients and progressive disease in three [Table 1]. This combination was well tolerated in the trial with a side effect profile similar to PARP inhibition alone. NCT04149145 is a phase I trial of niraparib in combination with the ATR inhibitor M4344 among patients with PARPi-resistant, recurrent ovarian cancer and was anticipated to begin enrollment in December 2022 (NCT04149145).

Table 1. Summary of key trials targeting ATR, CHK1, and WEE1 for the treatment of recurrent ovarian cancer

Target	Inhibitor	Trial Identifier	Trial Overview	Key Findings	Reference
ATR	Ceralasertib	NCT03462342	Phase II study of olaparib in combination with ceralasertib in platinum-resistant ovarian cancer	Among the 12 PARPi-naïve patients who were evaluable for response, the best response was stable disease in 9 patients.	[49]
CHK1	Prexasertib	NCT02203513	Phase II trial of prexasertib for recurrent ovarian cancer in patients without germline *BRCA*	Partial response rate of 29% (95% CI 13%-49%)	[64]
CHK1	Prexasertib	NCT02124148	Phase Ib study of prexasertib in combination with multiple chemotherapeutic agents in advanced cancer	No ovarian cancer-specific response data available Objective response rate of 12.7% in combination with cisplatin arm Hematologic toxicities were dose-limiting.	[65]
CHK1	Prexasertib	NCT03057145	Phase I study of prexasertib in combination with the PARPi olaparib in HGSOC and other solid tumors	Identified a schedule with acceptable tolerability Partial response rate of 22% in PARPi-resistant HGSOC	[66]
WEE1	Adavosertib	NCT01164995	Phase II study of adavosertib plus carboplatin in patients with *TP53*-mutant advanced ovarian cancer, platinum-resistant or -refractory	ORR of 43% Median PFS of 5.3 months Median OS of 12.6 months	[77]
WEE1	Adavosertib	NCT01357161	Phase II study of adavosertib versus placebo plus carboplatin and paclitaxel in patients with advanced, *TP53*-mutated platinum-sensitive ovarian cancer	Modest PFS prolongation in the combination arm: PFS (7.9 months *vs.* 7.3 months), HR 0.63 (0.38-1.06), P = 0.080) No significant change in ORR (74.6% *vs.* 69.4%, P = 0.52) or OS (HR 1.0 (0.53-1.88), P = 0.898) Increased adverse events in experimental arm	[78]
WEE1	Adavosertib	NCT02101775	Phase II randomized trial of adavosertib versus placebo with gemcitabine chemotherapy in patients with platinum-resistant or refractory HGSOC	Improved PFS in experimental arm (4.6 months *vs.* 3.0 months, HR 0.55 (0.35-0.90), P = 0.015) Improved OS in experimental arm (11.4 months *vs.* 7.2 months, HR 0.56 (0.35-0.91), P = 0.017) Improved partial response rate in experimental arm (23% *vs.* 6%, P = 0.038) Increased adverse events in experimental arm	[79]
WEE1	Adavosertib	NCT02272790	Adavosertib with chemotherapy in patients with primary platinum-resistant ovarian, fallopian tube, or peritoneal cancer: an open-label, four-arm, phase II study	The best ORR was 66.7%, with a disease control rate of 100%. Toxicity was notable. 100% of patients had grade 3 or higher adverse events.	[80]

ATR: Ataxia telangiectasia and Rad3 related; BRCA: breast cancer gene; CHK1: checkpoint kinase 1; CI: confidence interval; HGSOC: high-grade serous ovarian cancer; HR: hazard ratio; PARPi: poly (ADP-ribose) polymerase inhibitor; PFS: progression-free survival; ORR: overall response rate; OS: overall survival ratio.

CHK1

The serine/threonine kinase CHK1 plays an integral role in the cellular response to genotoxic stress, functioning as the principal effector for ATR. CHK1 has been described in multiple organisms to regulate cell cycle transition during basal states[50] and in response to DNA damage[51-53]. In addition, CHK1 regulates other proteins involved in DNA replication, including PCNA (proliferating cell nuclear antigen)[54] and Pol-α (holding cells at the G2 phase) [Figure 1][55]. Thus, activated CHK1 slows down DNA replication, allowing the cell to begin DNA repair, which is further enforced by CHK1 through its phosphorylation of Rad51, which is important in regulating HRR[56]. Given its role in cell cycle progression, CHK1 is heavily regulated, with phosphorylated CHK1 undergoing a transition from the nucleus to the cytoplasm and proteasome-mediated degradation[57].

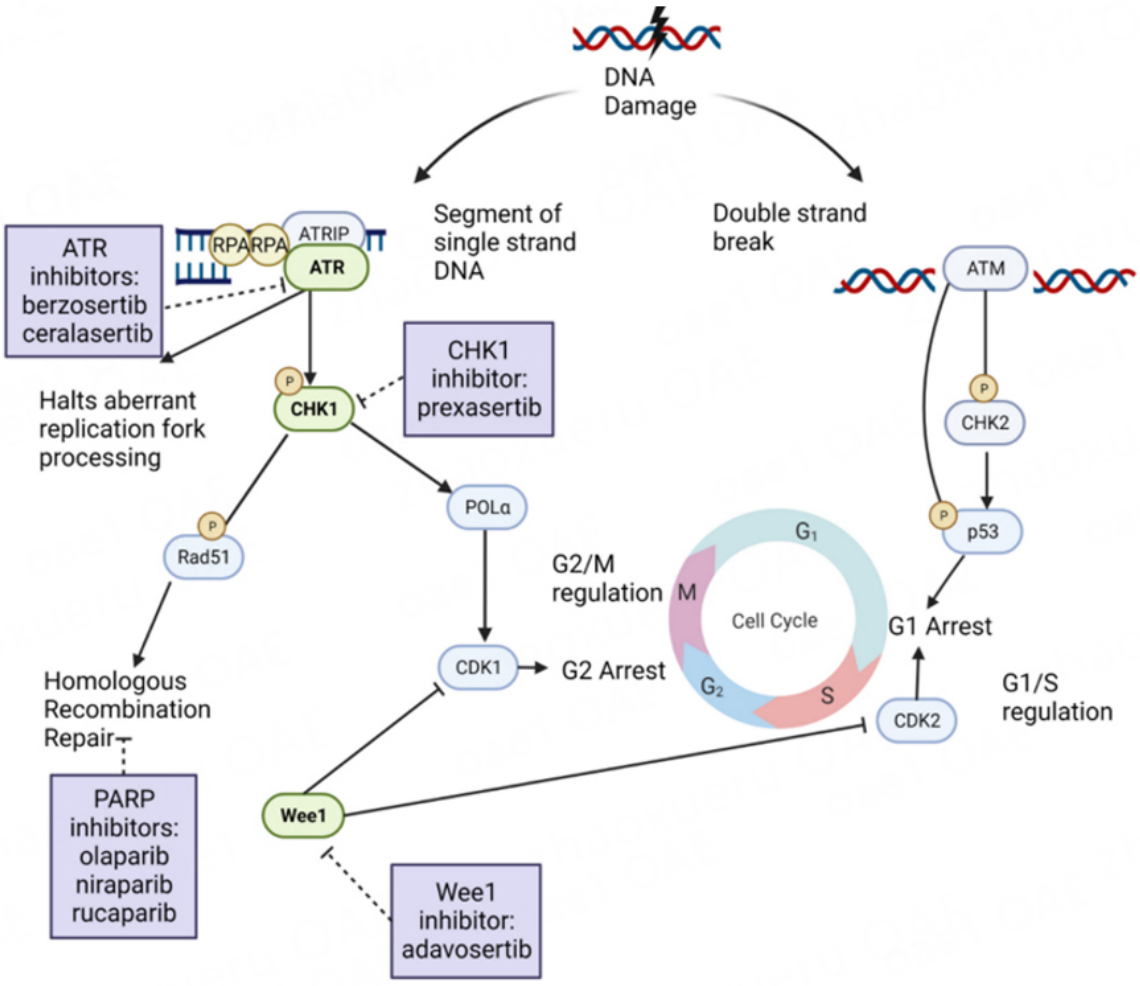

Figure 1. A schematic representation of the DNA damage response and its interaction with cell cycle regulation. RPA binds to sites of ssDNA and co-localizes with ATR and its binding partner ATRIP. CHK1 is the effector of ATR and regulates cell cycle progression via induction of G2 arrest. Ovarian cancer cells with deficient p53 function have aberrant G1/S regulation and rely more heavily on G2/M regulation. Wee1 activates the G2/M checkpoint through phosphorylation of CDK1, a critical regulator for cells with deficient p53 function.

The underlying hypothesis for targeting CHK1 stipulates that blocking CHK1 activity will promote cell cycle progression in the presence of DNA damage, resulting in an accumulation of double-stranded breaks in rapidly dividing cancer cells. This accumulated damage will ultimately lead to collapse of genomic integrity and cell death. HGSOC is a particularly good candidate for CHK1 inhibition because p53-deficient cells will be preferentially dependent on the G2-M checkpoint which may render them further susceptible to CHK1 inhibition[37,38].

To date, a majority of CHK1 inhibitors are ATP (adenosine triphosphate)-competitive and have been designed to have good selectivity over CHK1's highly homologous cousin CHK2 (checkpoint kinase 2)[58]. Multiple first-generation CHK1 inhibitors did not advance through early phase clinical trials due to unfavorable pharmacokinetic and pharmacodynamic profiles, as well as excess toxicity. However, a second-generation CHK1 inhibitor (CHK1i), prexasertib (LY2606368), has emerged as a prioritized compound for further development[59]. Prexasertib is a selective small molecule inhibitor of CHK1 and CHK2[60]. Prexasertib[61] showed efficacy as a monotherapy in HGSOC patient-derived PDX models which were

BRCA1-mutant or resistant to olaparib, with further synergy observed between a PARPi and CHK1i[62].

Prexasertib is currently advancing through clinical trials. It demonstrated favorable tolerability in a phase I clinical trial[63]. A phase II trial of prexasertib monotherapy for the treatment of recurrent HGSOC in patients without germline *BRCA* mutations demonstrated a partial response rate of 29% (95%CI: 13-49)[64], with an encouraging response rate in the platinum-resistant or platinum-refractory patient subgroup of 32%. Common treatment-emergent adverse events included neutropenia, leukopenia, thrombocytopenia, and anemia [Table 1][64]. These results are notable in this difficult-to-treat patient population and warrant further clinical investigation.

As described above, CHK1 is hypothesized to synergize with other agents that either induce DNA damage or inhibit its repair, such as chemotherapeutics and PARP inhibitors. A phase Ib study of prexasertib in combination with multiple chemotherapeutic agents (cisplatin, cetuximab, pemetrexed, or 5-fluorouracil) in patients with advanced or metastatic cancers reported an objective response rate of 12.7% in the cisplatin arm, with frequent and dose-limiting hematologic toxicities[65]. This approach has not been studied specifically in the platinum-resistant HGSOC population. A phase I study of prexasertib in combination with the PARPi olaparib in HGSOC and other solid tumors identified a schedule with acceptable tolerability and demonstrated preliminary activity in patients with *BRCA* mutations who had experienced prior progression on a PARPi [Table 1][66].

WEE1

WEE1 is a tyrosine kinase that activates the G2/M cellular checkpoint through phosphorylation and subsequent inhibition of CDK1 (cyclin-dependent kinase 1) and CDK2 (cyclin-dependent kinase 2), which regulate cell cycle progression in the presence of damaged DNA [Figure 1][67,68]. While normal cells repair damaged DNA during G1 arrest, malignant cells with deficient p53 and defects in the G1 checkpoint depend more on a functional G2–M checkpoint for DNA repair. Thus, inhibition of WEE1 can increase genomic instability and replication stress leading to mitotic catastrophe in cells with an overreliance on the G2/M checkpoint. Moreover, WEE1 levels are elevated in ovarian cancer[69]. Inhibition of WEE1 has also been shown to force cells arrested in S-phase into mitosis[70]. As such, WEE1 inhibitors have been hypothesized to synergize with DNA-damaging agents. This effect may be particularly relevant for p53-deficient cells, which are more dependent on the intra-S-phase checkpoint following DNA damage[70].

In 2009, Hirai *et al.* reported on the development of adavosertib (MK-1775, AZD1775), a potent and selective small molecule inhibitor of WEE1[71]. Treatment with adavosertib sensitized cells to the antitumor effects of chemotherapy, with the largest effects noted, as would be expected, in p53 deficient cell lines[71]. Additional preclinical studies confirmed the sensitivity of *TP53*-mutant cells to the combined effects of chemotherapy plus adavosertib[72-74] in non-ovarian cancer models, thus forming the basis for early phase I studies. Adavosertib was shown to negatively impact cell viability in both *in vitro* and *in vivo* models of ovarian cancer[75].

In clinical trials, adavosertib has been studied as a monotherapy or in combination with chemotherapy in advanced solid tumors[76]. Encouraging responses were noted in tumors with mutant *TP53*, leading to a follow-up phase II study in HGSOC, which harbors high rates of inactivating *TP53* mutations[33]. A phase II study of adavosertib plus carboplatin in patients with *TP53*-mutant advanced ovarian cancer, either resistant or refractory to first-line platinum-based chemotherapy, demonstrated an encouraging overall response rate (ORR) of 43% [Table 1][77]. Significant toxicities were noted, including frequent grade 3 or 4 thrombocytopenia and neutropenia.

A double-blind, randomized, phase II trial of the combination of adavosertib versus placebo plus standard platinum-based chemotherapy in patients with advanced, *TP53*-mutated platinum-sensitive ovarian cancer[78] failed to demonstrate a significant difference in objective response rate (74.6% *vs.* 69.4%, $P = 0.52$). A randomized phase II trial of adavosertib versus placebo with gemcitabine chemotherapy in patients with platinum-resistant or platinum-refractory HGSOC showed a modest improvement in PFS (4.6 months *vs.* 3.0 months, HR 0.55 (0.35-0.90), $P = 0.015$) but with increased toxicity in the combination arm [Table 1][79].

Most recently, Moore *et al.* reported on the results of an open-label four-arm phase II trial of adavosertib in combination with chemotherapy in patients with primary platinum-resistant ovarian, fallopian tube or primary peritoneal cancer[80]. The best ORR was 66.7% (disease control rate 100%), but toxicity was considerable in this arm, with 100% of patients experiencing grade 3 or higher adverse events [Table 1]. In addition to these studies demonstrating a promising role of WEE1 inhibitors in combination with chemotherapy, a number of trials [NCT02576444 (OLAPCO), NCT03579316] have been planned or are ongoing to investigate adavosertib in combination with PARP inhibitors due to the role of WEE1 in stabilization of replication forks[67]. This combination has shown synergy in preclinical models; however, toxicity concerns persist[81].

MORE SELECTIVE AND POTENT EXPLOITATION OF HRD

The first generation of PARP inhibitors (olaparib, niraparib, rucaparib, and talazoparib) inhibit both PARP1 and PARP2. It has been proposed that PARP1 inhibition is required to induce DNA damage[82]. Moreover, inhibition of PARP2 has been linked to suppression of erythropoiesis[83], and has been suggested to be a driver of the hematologic toxicity associated with the currently approved PARP inhibitors[84]. The first next-generation PARPi to enter clinical trials AZD5305 is a highly potent and selective PARP1 inhibitor[84]. AZD5305 is being studied in the ongoing PETRA study (NCT04644068), a phase I/II study of AZD5305 in patients with tumors harboring mutations in key DDR genes. Preliminary data demonstrated a favorable toxicity profile and encouraging clinical activity; publication of final results is highly anticipated. A PARP inhibitor with a superior therapeutic index may confer improved clinical activity as monotherapy. Moreover, an improved hematologic toxicity profile may allow for more effective and better tolerated combinations with other DNA damage repair inhibitors. Further clinical data are needed to evaluate these hypotheses.

BIOMARKERS PREDICTIVE OF RESPONSE

Konstantinopoulos *et al.* reported a post-hoc retrospective study investigating patients with HGSOC receiving gemcitabine with berzosertib (an ATR inhibitor) *vs.* placebo. They observed that the benefit from the addition of berzosertib was only seen in patients with a platinum-free interval of 3 months or less. They proposed that tumors with low replication stress (defined as the absence of CCNE1 amplification, RB1 two-copy loss, CDKN2A two-copy loss, KRAS amplification, NF1 mutations, ERBB2 amplification, MYC amplification, and MYCL1 amplification) preferentially benefit from the addition of ATR inhibition to chemotherapy[85]. Further validation of this proposed biomarker approach is still needed.

CCNE1(cyclin E1) amplification may be another potential predictive biomarker. CCNE1 forms a complex with CDK2, and when activated, this complex is an important regulator of the initiation of DNA replication. *CCNE1* amplification has been associated with an acceleration of progression through the G1/S restriction point in the cell cycle, ultimately leading to an increase in mutations in genes that control cell survival and proliferation[86]. *CCNE1* amplification has been proposed as a biomarker of intrinsic resistance to platinum-based chemotherapy in ovarian cancer[86]. More recently, *CCNE1* amplification has been proposed as a potential biomarker indicative of response to combined WEE1 inhibition and ATR inhibition in preclinical

ovarian cancer and endometrial cancer models. It has been proposed that *CCNE1*-overexpressing cells are preferentially dependent on the G2/M checkpoint. Both WEE1 and ATR are key regulators of the G2/M checkpoint, and as such, their inhibition may exploit a vulnerability in *CCNE1*-overexpressing cells that chemotherapy does not[87]. These results will require clinical validation, and perhaps more importantly, a combined WEE1-ATR inhibition strategy must demonstrate acceptable tolerability.

CONCLUSION

In this review, we have explored several approaches to overcome platinum-based chemotherapy resistance and PARPi resistance, focusing on the role of DNA damage repair inhibitors. Several DNA damage repair inhibitors have demonstrated activity, but efficacy in the platinum-resistant setting has been relatively modest. Moreover, considerable hematologic toxicity has been a recurring limitation of this approach. Work is being done to identify patients most likely to benefit from this treatment approach, and the possibility of combination treatment strategies with a next-generation PARPi holds some promise. Based on the available data, it is almost certain that additional treatment strategies will be needed to overcome resistance to first-line treatment more effectively.

Combination treatment strategies might be most effective if employed as part of first-line treatment to address intrinsic rather than acquired resistance to platinum chemotherapy and PARP inhibition. Moreover, we emphasize that the identified resistance mechanisms to both platinum-based chemotherapy and PARP inhibition rely on much more than the roles of ATR, CHK1 and WEE1 in the DNA damage response. For example, a recent study identifies a druggable nucleus-to-mitochondria reactive oxygen species (ROS) sensing pathway, which appears to mediate resistance to platinum-based chemotherapy in ovarian cancers[88]. The identification of a novel mechanism of resistance holds promise, but further research to identify its best clinical application is needed. Multimodal combination approaches incorporating newer classes of medications, including antibody-drug conjugates and novel immunotherapy constructs, hold promise as well.

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Author's contributions

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Immune checkpoint inhibitors in ovarian cancer: where do we go from here?

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Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy, and despite advancements in therapeutics, most women unfortunately still succumb to their disease. Immunotherapies, in particular immune checkpoint inhibitors (ICI), have been therapeutically transformative in many tumour types, including gynaecological malignancies such as cervical and endometrial cancer. Unfortunately, these therapeutic successes have not been mirrored in ovarian cancer clinical studies. This review provides an overview of the ovarian tumour microenvironment (TME), particularly factors associated with survival, and explores current research into immunotherapeutic strategies in EOC, with an exploratory focus on novel therapeutics in navigating drug resistance.

Keywords: Ovarian cancer, immunotherapy, tumour microenvironment, drug development

INTRODUCTION

Ovarian cancer is one of the most lethal cancers worldwide, with over 200,000 deaths in 2020[1]. Epithelial

ovarian cancer (EOC) is the most common subtype (90%), with germ cell tumours, sex cord-stromal tumours and other rare subtypes, including small cell carcinoma, accounting for the remaining 10%[2]. EOC (including fallopian tube and primary peritoneal cancer) is a complex disease as it encompasses a heterogenous group of inherently different histological subtypes with differing underlying genomic and molecular drivers resulting in different clinical behaviours and outcomes[3,4]. High-grade serous ovarian carcinoma (HGSOC) is the most common, followed by endometrioid carcinoma, clear cell carcinoma, low-grade serous carcinoma, and mucinous carcinoma[2].

Most women with EOC are diagnosed at advanced stage and more than 70% will recur within the first three years. *BRCA1/2* germline mutations are the strongest known genetic risk factors, with an estimated frequency of 13%-16% in women diagnosed with EOC[5,6,7], most of which are HGSOC histological subtype[7]. Germline *BRCA1/2* mutations are associated with greater sensitivity to platinum-based chemotherapy and poly(adenosine diphosphate[ADP]-ribose) polymerase inhibitors (PARPi), and carriers have generally been reported to have improved survival compared to non-mutation carriers with high-grade serous EOC[8,9].

The majority of EOC patients will eventually succumb to the disease, with 10-year survival being only 17%[10,11]. Cytoreductive surgery with chemotherapy remains the standard of care in the treatment of EOC[12]. Doublet-platinum chemotherapy can be administered as adjuvant treatment or as a neoadjuvant regime (usually three cycles prior to interval debulking surgery, followed by the remaining three cycles). Neoadjuvant chemotherapy provides an opportunity to assess chemosensitivity and assists with prognostication[13,14]. Other drug classes such as vascular endothelial growth factor (VEGF) inhibitors have shown survival benefits, and thus bevacizumab is routinely used in combination with chemotherapy as a maintenance strategy[15-17]. Most recently international, phase 3 studies have proven the effectiveness of PARPi in improving both progression and overall survival as well as the quality of life outcomes in both front-line and recurrent maintenance settings for certain subsets of advanced EOC, particularly those with *BRCA* mutations[8,9,18,19]. However, not all benefit from PARPi use, particularly those who are platinum-resistant, those without *BRCA* gene mutations and/or with intact homologous recombination repair (HRR) pathway, which comprises approximately 50% of the most common EOC histological subtype, HGSOC[3]. Unfortunately, even with PARPi, many patients will develop resistance and thus chemotherapy remains important in the treatment of recurrent ovarian cancer. With successive lines of treatment, most EOC will become resistant to chemotherapy, in particular to platinum-containing agents. Other subtypes such as low-grade serous, mucinous carcinoma and clear cell carcinoma are largely resistant to chemotherapy as a result of molecular and genetic profile and alterations[20]. Studies in preclinical models have suggested a number of mechanisms underlying acquired platinum resistance in EOC, including drug accumulation, drug efflux, cellular response to DNA damage and impaired apoptosis; however, evidence for clinical relevance for most of these mechanisms is lacking[21]. Limitations include using cell line models that are not representative of the common EOC subtypes seen clinically[22], and using non-physiological drug exposures to induce drug resistance phenotypes *in vitro*. More compelling studies investigating patient samples following the development of clinical resistance *in vivo* have implicated restoration of DNA repair pathways, including reversion of *BRCA* mutations and methylation, as significant mechanisms of acquired platinum resistance[23-25]. As the outlook for platinum-resistant EOC remains extremely guarded, with a median survival of only 12 months[26], there has been a strong focus on identifying mechanisms of resistance against platinum chemotherapy and PARPi, to address this urgent unmet therapeutic need. As such, many clinical trials are now standardly incorporating exploratory correlative studies with emerging and innovative technologies to further clarify molecular signalling events and proteomic characterisation of ovarian cancer to gain a deeper understanding of the disease, with the ultimate goal of developing and incorporating novel

therapeutics to avoid and/or bypass mechanisms of intrinsic and acquired resistance in EOC.

Immunotherapy has been transformative in the oncology therapeutic landscape. Immune checkpoint inhibitors (ICIs) counter several major immune-evasion mechanisms of cancer to induce killing of tumour cells by CD8+ T cells. Dramatic effects of improved progression-free and overall survival with utilisation of ICI have been demonstrated in a number of cancers, including melanoma, lung and colorectal cancers[27-29]. The durability and sustained benefit of ICI in other tumours make it an attractive therapy in ovarian cancer, particularly when there is evidence that ovarian cancer is immunogenic with the presence of tumour-infiltrating lymphocytes (TILs), regulatory T cells (Tregs), natural killer cells and tumour-associated macrophages (TAMs) detected in peripheral blood, ovarian cancer tissue and ascites[30-33]. Specific immune cell signatures, combined with multiple co-occurring DNA repair gene alterations and increased predicted neo-antigen load, have been associated with response to treatment and exceptionally long survival in patients with HGSOC, providing further evidence for tumour immune activity, at least in a proportion of cases[34].

However, disappointingly, durable responses to single-agent ICI in platinum-resistant EOC remain modest[35-37] and published studies to date have returned negative results with the addition of programmed cell death receptor-1 (PD1/L1) targeted ICI to standard adjuvant chemotherapy with or without bevacizumab[38,39]. Intriguingly, more encouraging clinical trial results from early phase studies exploring combination ICI with other agents such as PARPi, and VEGF inhibitors to act as immunomodulators have emerged.

In this article, the authors will describe the interplay between the immune system and tumour microenvironment (TME) including its interaction with the DNA damage repair (DDR) pathway, summarise the clinical trial landscape of immunotherapy in EOC, and discuss novel therapeutic pathways under investigation to overcome ICI therapy resistance.

ROLE OF THE TUMOUR MICROENVIRONMENT IN EPITHELIAL OVARIAN CANCER

As previously mentioned, ovarian cancer encapsulates a heterogenous group with subtypes that differ histologically, genomically and molecularly[3]. A dualistic model categorising EOC into two groups, Type I and Type II EOC, has been described[3]. According to this classification, Type I includes low-grade subtypes, typically arising from a recognisable precursor lesion such as borderline tumours with low malignant potential, whereas Type II EOC includes HGSOC, the most common subtype, with frequent *TP53* mutations and HRR pathway gene alterations[40]. Immune-cell infiltrates differ markedly between the histological subtypes, with the high-grade serous showing the most TIL infiltration, and the other subtypes being largely "cold" with respect to immune cell infiltrates, although there is wide variability between individual patients[41,42].

The defining genomic features of HGSOC are profound structural variation, including gene copy number alterations and genomic rearrangements, on a background of near-ubiquitous *TP53* mutation, with the most common somatic and germline alterations found in HRR pathways genes, mainly *BRCA1* and *BRCA2*[23]. When lacking HRR function, as in BRCA-mutant cells, DNA double-strand breaks will be processed by alternative but error-prone repair pathways, such as the non-homologous end joining repair (NHEJ), which lead to the accumulation of genomic instability and ultimately cancer cell death. NHEJ is faster than homologous recombination and mainly occurs in the G1 phase. Nevertheless, there is recent evidence that NHEJ functions throughout the cell cycle. Beyond the already-known proteins, such as Ku70/80, DNA-PKcs, Artemis, DNA pol λ/μ, DNA ligase IV-XRCC4, and XLF, new proteins are involved in the

NHEJ, namely PAXX, MRI/CYREN, TARDBP of TDP-43, IFFO1, ERCC6L2, and RNase H2. Among them, MRI/CYREN has a dual role, as it stimulates NHEJ in the G1 phase of the cell cycle and inhibits the pathway in the S and G2 phases[43]. In addition to this genomic complexity, a challenging clinical feature of HGSOC is the presence of widespread intraperitoneal disease at the time of diagnosis. Distinct spatial immunostimulatory and immunosuppressive mechanisms have been identified within individual patients depending on the anatomical site, with a relative paucity of immune cells in primary adnexal disease sites, in contrast with metastatic sites[44].

The impact of the heterogeneity of the tumour microenvironment on response to immunotherapy between and within ovarian cancer patients is not yet fully understood.

The immune system plays an integral and extensive role in tumour formation and growth in all tumours, including ovarian cancer [Figure 1]. Tumour growth is facilitated by the critical interplay between TME and immune cells, including natural killer (NK) cells, CD8+ T cells and CD4+ helper T (Th) cells together with pro-inflammatory macrophages (M1) and dendritic cells. When a tumour is formed, it is recognised and eliminated by the immune system (elimination phase). The tumour cells which survive this process enter a phase called equilibrium phase, during which the tumour can continue to grow despite ongoing destruction from the immune system. Eventually, tumour cells evade the immune recognition and destruction, termed the escape phase, leading to continual tumour growth and progression and finally metastasis. TME is a collective term that encapsulates a dynamic interplay between tumour cells and components of the immune system and plays an important role in tumour differentiation, dissemination, and immune evasion. It consists of extracellular matrix consisting of matrix metalloproteinases (MMPs) and stromal cells including tumour-infiltrating lymphocytes (TILs), fibroblasts, and endothelial cells, amongst others.

Tumour-infiltrating lymphocytes

TILs are white blood cells including T cells, B cells, macrophages and natural killer cells, which are localised to tumour or tumour stroma in response to molecular signals. CD3+, CD4+ ,and CD8+ TILs have long been known to exist in ovarian cancer, and an association between the presence of TILs and improved overall survival (OS) in ovarian cancer has been demonstrated in multiple studies. A meta-analysis to evaluate the prognostic value of TILs in ovarian cancer has found a significant association between TILs and survival, with a hazard ratio of 2.24 for those without TILs[45]. A study of 5577 EOC tissue samples identified that CD8+ TILs were associated with longer OS for those with multiple histological subtypes, particularly those with high-grade serous ovarian carcinomas. The median OS survival was 2.8 years for those with no CD8+ TILs compared to 5.1 years in those with high levels regardless of the extent of residual disease post cytoreductive surgery, standard treatment, and germline *BRCA1* mutation[41]. It also demonstrated prognostic value in other histological subtypes including endometrioid and mucinous carcinomas. Overall, CD8+ TILs have become the standard for prognostic evaluation for TILs in ovarian cancer.

CD8+ T lymphocytes

When naïve T cells are activated, they differentiate into CD8+ cytotoxic T cells and are migrated into TME via a number of signalling proteins called chemokines (including CCL5, CXCL9, CXCL10)[46]. CD8+ cytotoxic T cells target tumour cells via T cell receptor interaction with MHC Class I, facilitating tumour apoptosis via several mechanisms including perforin and granzyme B secretion. Tumour cells adopted numerous mechanisms to evade apoptosis, such as downregulation of MHC Class I, dysregulated expression of death receptors, or modification of antigen processing and thus reduced presentation capacity[47]. Additionally, CD8+ cytotoxic T cells upregulate checkpoint receptors such as PD-1, CTLA-4, LAG-3 and TIM3, which makes them susceptible to inhibitory signalling since binding of checkpoint receptors induces cytotoxic T cell exhaustion and anergy[47,48].

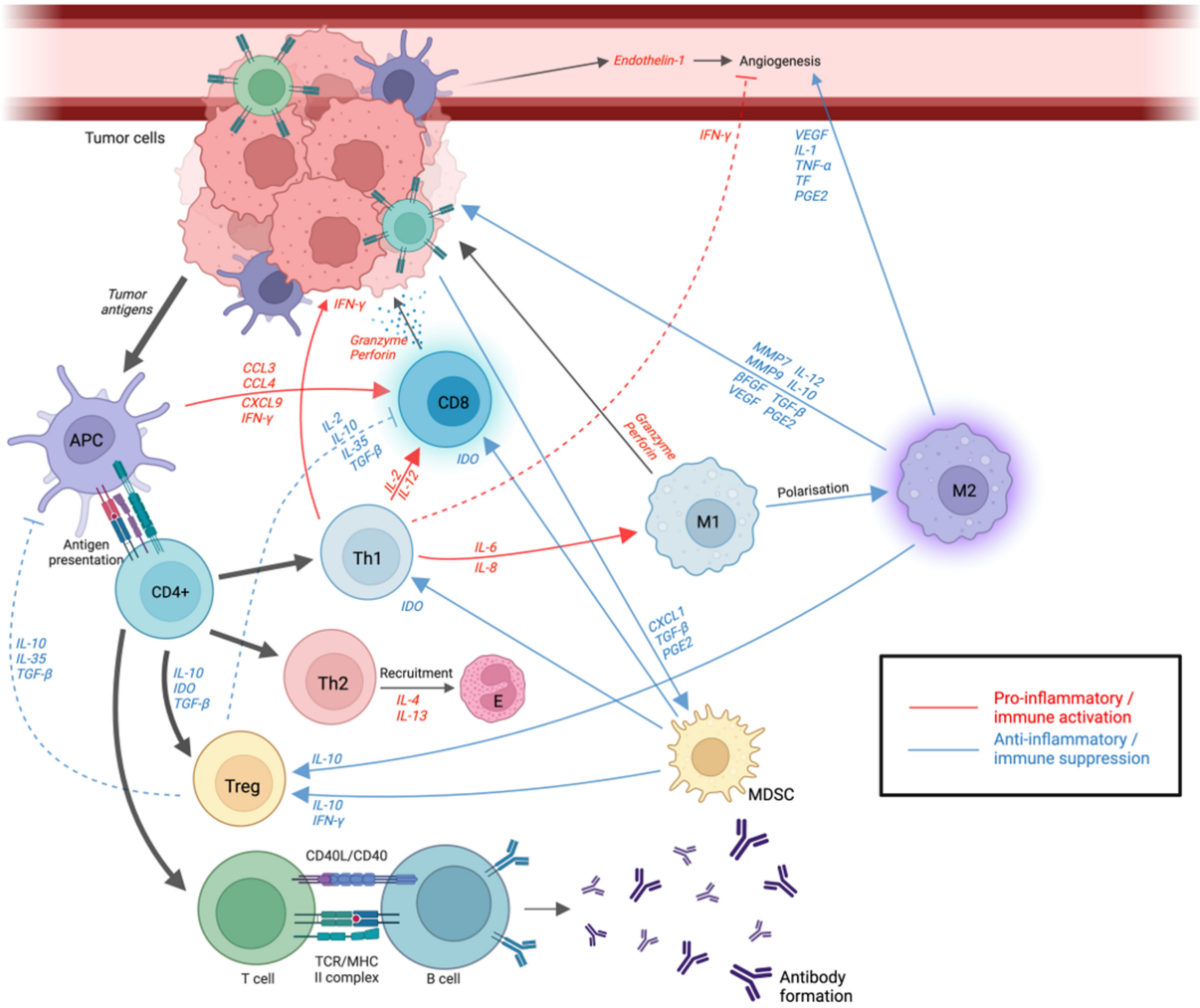

Figure 1. Schematic diagram outlining complex pro- and anti-inflammatory interplay within epithelial ovarian cancer microenvironment. Created with Biorender.com. Tumour cells recognised by APC, from which tumour antigens are presented to naïve CD4+ T cells, triggering T cell differentiation into Th1, Th2, Treg cell subclones (and other Th subsets – not pictured). Cytotoxic CD8+ T cells are recruited to TME via chemokines such as CCL3 and CXCL9 and are activated. Once activated, it releases granzyme and perforin to lyse cells directly. Th1 cells have dual action by secreting cytokines including IL-2 and IL-12, further activating CD8+ T cells to directly kill tumour cells as well as secreting IFN-gamma to suppress angiogenesis. Th2 assists eosinophil recruitment via IL-4 and IL-13 cytokine release, which assist in antitumour response. Treg cells suppress DC maturation and mute CD8+ T cell response by secreting inhibitory cytokines IL-10, IL-35, and TGF-β in response to regulate inflammatory response. M1 TAM, a pro-inflammatory type, is involved in tumour destruction, while M2 TAM exhibits immunosuppressive effects allowing tumour migration and invasion by releasing factors including endothelin-1, VEGF and TNF-alpha to induce tumour angiogenesis. $CD4^+$ T cells bind to B cells via major histocompatibility II complex to induce antibody formation via the adaptive immune system. APC: antigen-presenting cell; MDSC: Myeloid-derived suppressor cells; IL: Interleukin; TGF-β: transforming growth factor-beta; TNF: Tumour necrosis factor; CCL: Chemokine (C-C Motif); CXCL: Chemokine (C-X-C Motif); IDO: Indoleamine 2,3-dioxygenase; Th: T-Helper; M1 and M2 TAM: Tumour-associated macrophages; MMP: matrix metalloproteinase; E: eosinophil; VEGF: vascular endothelial growth factor; TF: tissue factor; PGE2: prostaglandin E2; IFN: interferon; β-FGF: beta-fibroblast growth factor.

CD4+ T lymphocytes

Naïve CD4+ T cells are activated by tumour antigens, and they differentiate into different subsets, including Th1, Th2, Th9, Th17, Th22, and Tregs. While the majority of CD4+ lymphocyte subsets further assist the activation of CD8+ T lymphocytes, thus facilitating the killing of the tumour, some have dual roles in promoting tumour growth via increasing angiogenesis.

T regulatory cells

T regulatory cells (Tregs) play an important role in suppressing immune responses and maintaining self-tolerance. Tregs in ovarian cancer are identified by several marker expressions, CD4 and CD35, which are present extracellularly, or forkhead box P3 (FOXP3) located intracellularly. When activated, Tregs release inhibitor cytokines such as TFT-β and IL-10 facilitating immunosuppressive effects on TME[49]. Increased Treg presence has been identified in tumours that are able to evade immune destruction, and it has been demonstrated that increased recruitment of Treg cells is associated with reduced survival with a high death hazard ratio in ovarian cancer[45,48]. Those who have undergone primary debulking surgery for ovarian cancer were identified to have decreased Tregs and increased TILS compared to those with suboptimal debulking surgery had the opposite trend[50,51]. Additionally, those undergoing neoadjuvant chemotherapy had lower FOXP3+ Treg infiltration and demonstrated higher survival compared to those with higher FOXP3 Treg counts[52]. Although Treg is not recognised as a prognostic factor, it seems clear that Tregs in TME have immunosuppressive effects, thus hampering the immune system to destroy cancer cells.

Myeloid-derived suppressor cells

Myeloid-Derived suppressor cells (MDSCs) consist of a heterogenous population of myeloid cells expressing GR-1 and CD11b myeloid surface markers. MDSCs mostly act to suppress the immune system via T cells in multiple ways, eventually promoting tumour progression. MDSCs reduce essential amino acids such as L-arginine and L-cystine required for T cell activation and function[53], and also inhibit the recruitment of T cells and promote T cell apoptosis. MDSCs also promote Treg cell activation and stimulate oxidative stress as well as facilitate neovascularisation, therefore priming and promoting tumour progression[53,54].

Dendritic cells

Dendritic cells (DCs) play a critical role in bridging the innate and adaptive immune systems and are essential in precipitating T cell immune response. As antigen-presenting cells, DCs facilitate tumour antigen recognition and presentation, thus triggering appropriate T-cell response. DCs also play an important role in activating and manipulating cytotoxic T-lymphocyte population in the TME mostly mediated by appropriate DC maturation. When DC maturation process becomes faulty due to various mechanisms, it can result in a more tumour-tolerant TME, thereby promoting tumour progression.

Programmed cell death ligand-1

Up to 60% of EOC express PD-L1, which has been identified as a poor prognostic factor for both PFS and OS[37-39,55,56]. In a study by Hamanishi *et al.*, five-year survival rates were found to be 80.2% *vs.* 52.6% for high and low PD-L1 expressing EOC, respectively[56]. Furthermore, CD8+ TILs appear to be inversely correlated to PD-L1 expression[56]. PD-1 is a surface molecule commonly expressed on CD8+ T cells and is a negative regulator of T cell activation. Its expression blocks entry into the cell cycle and production of cytokines such as IFN-gamma and TNF-a involved in activating inflammatory and immune responses[57]. Upregulation of PD-L1, which is exclusively expressed on the surface of tumour cells and a common ligand for PD-1, causes impaired tumour destruction and apoptosis of T cells. PD-1 pathway activation also escalates Treg function[58]. Thus, collectively activation of the PD-1/L1 pathway collectively results in the ability of cancer cells to evade the immune system and facilitating ongoing tumour growth. Harnessing and manipulating this pathway has been the rationale for therapeutic use for PD-1/L1 targeted ICIs. Despite the tremendous efficacy and clinical benefit of anti-PD-1/L1 ICI in other tumour types, it has been largely disappointing in ovarian cancer.

IMMUNE CHECKPOINT INHIBITORS: A WORK IN PROGRESS

Current understanding of immune-mediated response in relation to malignancy and tumour microenvironment has exponentially increased over the last two decades, especially with the development of immune checkpoint inhibitors. ICIs are now being commonly utilised in the treatment of both non-solid and solid malignancies, with efficacy rates ranging from 70% in lymphoma[59] and 39%-60% well-selected solid tumour groups, including those with high microsatellite instability (MSI-H)[60,61]. Impressively, in some cohorts, such as metastatic melanoma, 22% and 19% were able to achieve complete response with doublet and single-agent ICI, respectively[27]. Additionally, the durability of ICI makes it an attractive therapeutic, with overall survival of 44%-52% in metastatic melanoma cohorts at five-year follow-up noted[27]. Unfortunately, these drugs are not without their side effects. While reasonably well tolerated, grade ≥ 3 treatment-related adverse events occur in approximately 20%-25% of cases, and this rate can increase to 59% with the use of doublet ICIs[27,28]. While most immune-related toxicities are easily manageable, they are often life-long issues that require constant monitoring and management; thus, careful selection of patients for immunotherapy is paramount[27]. Additionally, the cost of ICI is problematic, as most Western and first world countries can fund it via government-subsidised programs or medical insurance; generalisability and utility of ICI worldwide realistically will be challenging. Particularly in low-middle-income countries, financial toxicity is a real issue affecting the quality of life for many patients affected by cancer.

Despite the significant advances in the understanding of the role of TME in EOC, the efficacy of single-agent and combination ICI therapy has been somewhat disappointing, as demonstrated through numerous international randomised trials. Amongst tumour types where immunotherapy is typically effective, there have been therapeutic strategies posited to transform "cold" tumours to "hot", that is, by increasing T-cell infiltration to predispose tumour cells to immune therapy anti-cancer effects[62]. Given the current therapeutic landscape of ovarian cancer and the limited role of immunotherapy, ongoing clinical trials are focused generally on three mechanisms: overcoming resistance by administering ICI therapy in synergism with other drug classes, enhancing immune responses through utilising other immune pathways, or bypassing resistance through exploring alternative therapeutic pathways [Figure 2]. Biomarker studies will provide further crucial information in determining which patients will benefit from particular treatment strategies. This section outlines the state of evidence for ICI therapies in EOC and discusses combination trials in progress looking to overcome resistance.

Single-agent ICI therapy

Several PD-1/L1 targeted ICI monotherapy trials exist in advanced or recurrent EOC, most of which demonstrated modest efficacy. KEYNOTE-100 study, a phase II trial which included a total of 376 advanced recurrent EOC patients administered 200 mg of pembrolizumab, 3-weekly[63]. It demonstrated only a modest ORR of 9.9% with a suggestion of the trend of increased responses for those with higher PD-L1 expression measured by combined positive score (CPS). The median PFS was 2.1 months, while the median OS was 18.7 months. Aside from CPS, no other clinical features such as histology or platinum-sensitivity display a correlation with higher response.

Numerous other studies investigating different single-agent anti-PD-1/L1 ICI agents such as avelumab and nivolumab in recurrent and platinum-resistant disease have demonstrated similarly disappointing modest responses with ORR[64-66]. Despite the initial suggestion of higher response for clear cell carcinomas, this has been largely disappointing, with no clinically meaningful ORR or survival benefit in the recently presented MOCCA trial[67]. Additionally, JAVELIN Ovarian 100[38] and IMagyn050[39] incorporating ICI with standard chemotherapy in the upfront setting have demonstrated no significant benefits, without improvement in PFS or OS, despite PD-L1 stratification[38,39].

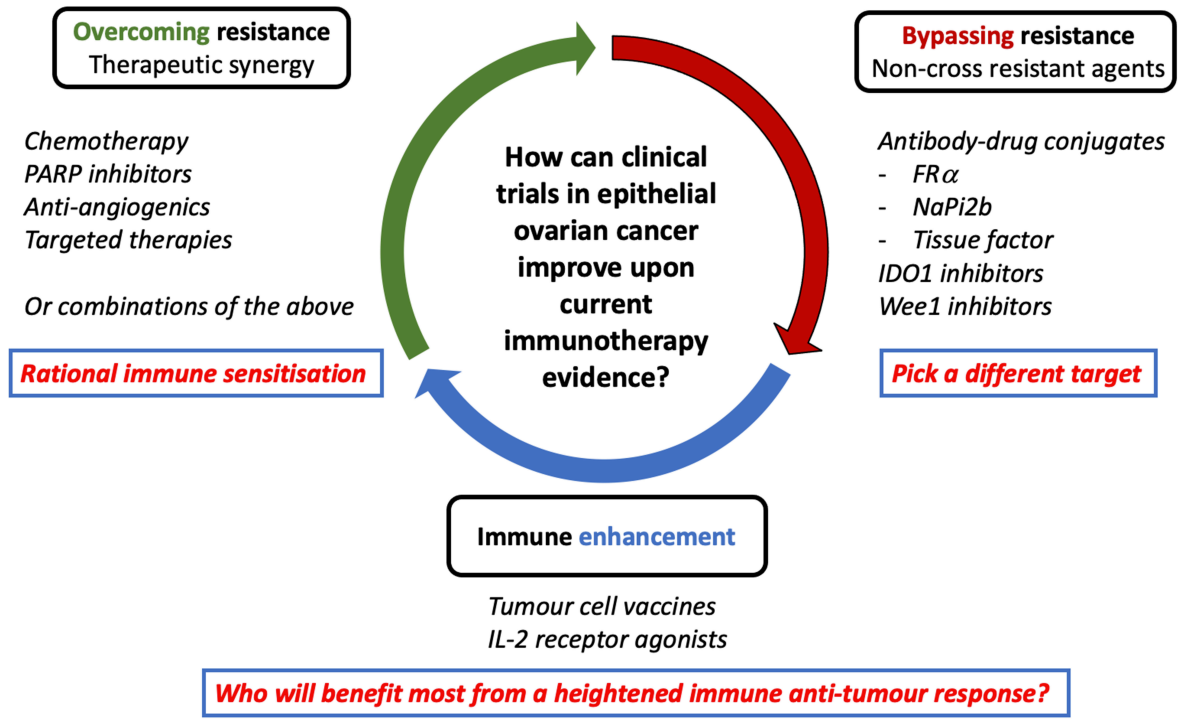

Figure 2. Schema outlining biomarker-driven strategies to improve upon current evidence for immunotherapy in ovarian cancer. Current trials are focused on improving clinical outcomes by either overcoming resistance through synergistic therapeutics (rational immune sensitisation) or bypassing resistance mechanisms (choosing different therapeutic targets). FRα: folate receptor-alpha; IDO1: Indoleamine 2, 3-dioxygenase 1; PARP: Poly-ADP ribose polymerase.

Despite disheartening single-agent anti-PD-1/L1 ICI results across the board of different EOC subtypes, a number of different studies examining different therapeutic combinations with ICI have been undertaken to investigate whether drug synergism of ICI can be enhanced for advanced EOC.

Dual-agent ICI therapy

Ipilimumab is a monoclonal antibody targeting cytotoxic T-lymphocyte associated protein-4 (CTLA-4), another immune checkpoint surface protein involved in negatively regulating T-cell function in priming immune response. Combining anti-CTLA4/anti-PD1 inhibitors have demonstrated significant treatment effects in other solid cancers, although this efficacy is limited in EOC. Zamarin *et al.* demonstrated that from the 100 recurrent EOC patients, those who had doublet-ICI demonstrated higher ORR at 6 months (31.4% *vs.* 12.2%) and PFS improvement (HR 0.528, 95%CI: 0.339-0.821; $P = 0.04$)[68]. Again, there was no association between the magnitude of PD-L1 staining and other clinical outcomes, which suggests the need for better predictive biomarkers to assist with ICI usage guidance. As expected, grade 3 adverse events were higher in the combination group (49% *vs.* 33%), although this did not reach statistical significance.

Combination studies with antiangiogenic agents

Angiogenesis is a hallmark of cancer growth and metastasis, and VEGF plays an integral role in this process. It binds to a number of VEGF receptor tyrosine kinases, namely VEGF-A, VEGF-B, VEGFR-C/D, VEGFR-1/2, and VEGFR-2/3. Amongst solid cancers, EOC is known to have high VEGF expression[69] and the use of bevacizumab, a VEGF-A inhibitor, is commonplace with proven clinical benefit in both platinum-sensitive and platinum-resistant settings[17,70]. Furthermore, there is strong evidence demonstrating the immunomodulatory effects of VEGF; preclinical data demonstrate that signalling through VEGFR-1

mediated by VEGFR-A can suppress maturation of dendritic cells, increase Treg population via VEGFR-2 signal and stimulate the growth of myeloid-derived suppressor cells, leading to further suppression of TME[71,72]. VEGF blockade leads to increased cytotoxic T cell recruitment and migration to tumour cells with a reduction in CD4+ Treg in TME[50,73,74]. Therefore, the potential synergistic activity of ICI therapy with antiangiogenic therapy has been hypothesised.

In addition to the previously discussed IMagyn050 in the upfront setting, nivolumab and atezolizumab combined with bevacizumab have been investigated in the recurrent setting[68,75]. Liu *et al.* recruited 38 women with recurrent, pre-treated EOC in a phase II study in which the ORR was higher than single-agent ICI with 28.9%; however, disappointingly, the majority of the benefit appeared to lie within the platinum-sensitive cohort with a median PFS of 12.1 months versus 7.7 months in the platinum-resistant cohort[76]. These findings again highlight the importance of patient selection for these therapies, although at present, aside from platinum sensitivity, more accurate and pertinent biomarkers are yet to be discovered to guide therapy.

Other antiangiogenic therapies have also been explored in combination with ICI therapy including lenvatinib and sitravatinib. Lenvatinib is a multi-receptor tyrosine kinase inhibitor, which mainly targets VEGF 1-3, fibroblast growth factor (FGF) receptors 1-4, platelet-derived growth factor receptor a, KIT, and RET. LEAP-005[77], an ongoing multi-cohort phase II study, includes 31 recurrent EOC patients with three or more prior lines of treatment including bevacizumab (ClinicalTrials.gov, NCT02501096). The results were more encouraging - ORR was 32% with a median PFS of 4.4 months, and nearly a quarter of responders were platinum resistant. Sitravatinib, an orally available multi-tyrosine receptor inhibitor that acts on VEGF2, KIT and TAM receptors, has also been identified to modulate immunosuppressive influences on TME. An early-phase study investigating tislelizumab, an anti-PD-1 monoclonal antibody, with sitravatinib in 60 platinum-resistant EOC demonstrated an ORR of 26% with a median PFS of 4.1 months and a median OS of 12.8 months[78]. Unfortunately, these novel combination therapies report significant treatment-related adverse events requiring dose reductions or interruptions in significant proportions of patients. Although these combinations are promising in overcoming therapeutic resistance in recurrent EOC, issues of tolerance and quality of life remain important factors. Lastly, AK112, a novel anti-PD-1/VEGF-A bi-specific antibody, has been investigated in an ongoing Phase Ia/Ib study in platinum-resistant/refractory EOC[79]. The ORR was higher at 29.4%, with two spectacular responders noted: one with clear cell histology with prior ICI exposure and one with HGSOC. The toxicity profile was similar to sitravatinib, but tolerability was much better with manageable adverse events in comparison.

In summary, trials so far investigating ICI and angiogenic combination approach have demonstrated preliminary efficacy, which appears to be clinically meaningful, but balancing this with the challenge of managing adverse events represents the true dilemma in moving forward, particularly in terms of patient selection for therapy, appropriateness of dose reductions, and ongoing biomarker discovery. As the role of combination angiogenesis and immunotherapy agents grows in EOC treatment, clear guidelines on practical aspects and real-world data will become increasingly relevant in informing clinicians of best practices.

Combination studies with PARP inhibitors

PARPi, namely olaparib, niraparib and rucaparib, have demonstrated breakthrough improvements in survival outcomes for individuals with EOC. Defects in the HRR pathway are strongly associated with the development of high-grade serous and endometrioid ovarian cancers, and it is in these patients with demonstrable HRR deficiencies or mutations that PARPi confer the greatest clinical benefit. Unfortunately,

a substantial proportion of individuals develop resistance to PARPi, and it is in this population that therapeutic direction remains unclear. Named resistance mechanisms to PARPi in the literature include drug efflux transporters, decreased PARP trapping, replication fork stabilisation and restoration of HR repair in multiple ways including alteration of PARP1, reversion mutation in HRR genes, and loss of *BRCA1* promotor methylation[80]. Of these, HR reversion mutations and drug efflux transporters (such as *ABCB1* mutations) have been implicated most prominently in the literature[81-87].

PARPi are inherently immunogenic by the mechanism of action, leading to a consequent increase in genomic instability and formation of neoantigens leading to immune detection via T cells[88]. Additionally, there is evidence to suggest that *BRCA1* deficiency may induce STING-dependent immune response by inducing type I interferon and pro-inflammatory cytokine production, providing some interconnected relationship between the therapeutic effect of PARPi and the immune response[89]. It has also been demonstrated that PARP inhibition inactivates GSK3 and upregulates PD-L1 in a dose-dependent manner in various cell lines, such as breast and pancreatic cells[90,91]. Consequently, T-cell activation is suppressed, resulting in enhanced cancer cell apoptosis[90]. While the complete immunomodulatory effect of PARPi on TME is not yet fully explained, several studies have shown promising synergistic effects. TOPACIO, a phase I/II study, examined niraparib with pembrolizumab in 62 patients with recurrent platinum-resistant or -refractory EOC[92]. The ORR was 18%, with the most common treatment-related adverse events being grade 3 anaemia and thrombocytopaenia. No correlation in efficacy with *BRCA* and HRD status, PD-L1 expression and prior bevacizumab was observed. While it did not meet its predefined endpoint, the combination showed meaningful activity compared with single agent-based therapy in the same population with resistant or refractory OC. Another study examined olaparib with durvalumab in 32 platinum-sensitive EOC patients with germline *BRCA1/2* mutations and 31 *BRCA* wild-type patients with the addition of bevacizumab[93]. The ORR was 71.9% for olaparib and durvalumab in *BRCA* mutated cohort, 34.4% in *BRCA* wild-type patients, and 87.1% in the triplet arm *vs.* 34.4% with doublet therapy. Mean PFS was also longer with triplet therapy (14.7 *vs.* 5.5 months). Overall, both triple and doublet therapy was well tolerated, with adverse events reported consistent with those previously reported for single drugs, and discontinuation of one or more drugs as a result of adverse events was greater with triplet therapy (16% *vs.* 6%).

Currently, there are conflicting results seen in trials investigating triplet therapy consisting of PARPi, antiangiogenics, and ICI. Zimmer *et al.* conducted a trial of cediranib, an oral anti-VEGFR1-3 agent combined with durvalumab and Olaparib, in 35 recurrent EOC patients with promising preliminary results of an ORR of 44%[94]. A phase II is currently underway, with results expected in 2025. Meanwhile, other two-phase II studies, OPAL and GINECO BOLD, demonstrated a lower-than expected-ORR of 17.9% and no benefit in PFS and OS in either platinum-resistant or platinum-sensitive cohort[95,96]. Eventually, when results from larger combination studies (demonstrated in Table 1) become available, careful comparisons between triplet therapy studies will need to be considered, particularly in terms of patient selection, rates of toxicity and efficacy outcomes, especially as specific drug combinations can have varying synergistic effects. This is especially applicable to the trials in the first-line setting, where improvements in PFS and OS are expected to be highest in the chemotherapy-naïve setting.

The AMBITION study was the first biomarker-driven, targeted trial in heavily pre-treated platinum-resistant EOC, which included 70 patients[97]. The patients were allocated to receive combination therapy based on HRD and PD-L1 status determined on archival tumour sample. The patients were randomised to either olaparib and cediranib arm or olaparib or durvalumab arm. For HRD-positive patients or for HRD-negative patients, they were allocated to either durvalumab and ChT (either pegylated liposomal doxorubicin or topotecan or weekly paclitaxel) or durvalumab and tremelimumab and ChT. PD-L1

Table 1. Summary of ongoing immune checkpoint inhibitor combination trials in epithelial ovarian cancer. Taken from clinicaltrials.gov. (Accessed on 9th of January, 2023)

Trial Identifier	Trial name	Phase	Setting	*n*	Treatment Arm	Endpoints	Expected completion
NCT04417192	Olaparib Monotherapy and Olaparib + Pembrolizumab combination Therapy in Ovarian Cancer (OLAPem)	II	Upfront HRD positive only	30	Cohort 1: Olaparib Cohort 2: Olaparib, Pembrolizumab	ORR* AE CRS PFS OS	December 2023
NCT03740165	Study of chemotherapy with pembrolizumab (MK-3475) followed by maintenance with Olaparib (MK-7339) for the first-line treatment of women with BRCA non-mutated advanced epithelial ovarian cancer (EOC) (MK-7339-001/ KEYLYNK-001/ ENGOT-ov43/GOG-3036	III	Upfront	1367	Pembrolizumab, Olaparib + Chemotherapy (carboplatin/paclitaxel) + Bevacizumab	PFS* OS OS (PD-L1 CPS > 10) PFS2 AE QoL TTD pCR	May 2025
NCT03737643	Durvalumab treatment in combination with chemotherapy with bevacizumab, followed by maintenance durvalumab, bevacizumab and olaparib treatment in advanced ovarian cancer patients (DUO-O)	III	Upfront	1374	Durvalumab, Olaparib + chemotherapy (carboplatin/paclitaxel) + bevacizumab	PFS* OS PFS2 QoL pCR ORR DOR	May 2028
NCT03522246	A study in ovarian cancer patients evaluating Rucaparib and Nivolmab as Maintenance treatment following response to front-line platinum-based chemotherapy (ATHENA)	III	Upfront	1000	Rucaparib, Nivolumab	PFS* OS ORR DOR	December 2030
NCT03602859	A phase 3 comparison of Platinum-based therapy with TSR-042 and niraparib versus standard of care (SOC) platinum-based therapy as first-line treatment of stage III or IV non-mucinous Epithelial Ovarian Cancer (FIRST)	III	Upfront	1405	Dostarlimab, Niraparib +/- chemotherapy (carboplatin/paclitaxel)	PFS* OS AE QoL	June 2026
NCT04679064	Trial of Niraparib-TSR-042 (dostarlimab) *vs.* Physician's choice of Chemotherapy in recurrent, ovarian, fallopian tube or peritoneal cancer patients not candidates for platinum retreatment (NItCHE-MITO33)	III	Recurrent	427	Arm A: Dostarlimab, Niraparib Arm B: Chemotherapy (pegylated liposomal doxorubicin, paclitaxel, gemcitabine, topotecan, bevacizumab)	OS* PFS ORR AE PRO	January 2025
NCT03598270	Platinum-based chemotherapy with Atezolizumab and Niraparib in patients with recurrent ovarian cancer	III	Recurrent	414	Niraparib + chemotherapy (gemcitabine, carboplatin, paclitaxel, pegylated liposomal doxorubicin) + /- atezolizumab	OS* PFS PFS2 AE PROs ORR DOR	January 2025
NCT04742075	Olaparib, Durvalumab and UV1 in relapsed ovarian cancer (DOVACC)	II	Recurrent	184	Arm A: Olaparib	ORR*	June 2026

					Arm B: olaparib, durvalumab Arm C: Olaparib, durvalumab, UV1	PRO ORR Safety	
NCT05231122	Pembrolizumab combined with bevacizumab with or without agonist Anti-CD40 CDX-1140 for the treatment of recurrent ovarian cancer	II	Recurrent	80	Pembrolizumab, bevacizumab, CDX-1140 (anti-CD40 agonist monoclonal antibody)	AE* ORR* PFS OS DCR QoL	January 2026
NCT04781088	Lenvatinib, Pembrolizumab, and Paclitaxel for treatment of recurrent endometrial, epithelial ovarian, fallopian and peritoneal cancer	II	Recurrent	38	Pembrolizumab, Lenvatinib, paclitaxel	ORR* AE PFS	February 2025
NCT03206047	Atezolizumab, Guadecitabine, and CDX-1401 Vaccine in treating patients with Recurrent Ovarian, Fallopian tube and peritoneal Cancer	II	Recurrent	75	Atezolizumab + Guadecitabine + CDX-1401 vaccine	AE* PFS* OS ORR CA125 reduction DOR	March 2023
NCT02873962	A phase II study of Nivolumab/ Bevacizumab/Rucaparib	II	Recurrent	76	Nivolumab, bevacizumab, rucaparib	ORR* AE* PFS OS DOR	June 2024
NCT05092360	Phase III study of Nemvaleukin alfa in combination with pembrolizumab in patients with platinum-resistant epithelial ovarian cancer (ARTISTRY-7)	III	Recurrent	376	Arm A: Nemvaleukin-alfa plus pembrolizumab Arm B: Nemavaleukin-alfa Arm C: Pembrolizumab Arm D: Physician's choice chemotherapy	PFS* ORROS DCR DOR TTR CA125 AE	December 2026

ORR: Objective response rate; CRS: chemotherapy response score; PFS: progression-free survival; OS: overall survival; PRO: patient-reported outcomes; TTD: time to deterioration; pCR: pathological complete response; DOR: Duration of Response; AE: adverse events; CA125: Cancer Antigen 125; QoL: quality of life; PFS2: progression-free survival to second therapy; PD-L1: programmed cell death ligand 1; CPS: combined positive score; TTR: time to response. *primary endpoint.

positivity was defined as 25% or more of expression via Ventana SP263 assay. The overall ORR was 37.1%, with the highest ORR of 50% observed in Olaparib-cediranib cohort, closely followed by olaparib-durvalumab cohort with an ORR of 42.9%, as excepted for HRD-negative cohorts, which had a low ORR ranging between 20%-33%. All treatment groups were manageable, with no treatment-related adverse events leading to discontinuation.

Currently, there are many ongoing trials of ICI with different combinations in EOC. These are listed in Table 1, although the list of trials included is not exhaustive.

NOVEL TARGETS AND IMMUNE ENHANCEMENT IN OVARIAN CANCER

Unfortunately, it remains unclear as to whether ICI therapies will prove successful in improving clinical outcomes in EOC, and investigation of several novel therapeutic targets is currently underway.

Antibody-drug conjugates

Structurally, antibody-drug conjugates (ADC) are comprised of a targeted monoclonal antibody attached to a cytotoxic payload through a linker molecule, where binding of the antibody to its target results in endocytosis and subsequent cytotoxic action in cancer cells[98]. Several molecules have shown promise in the treatment of EOC, as discussed below, although they remain highly variable in efficacy and rates of toxicity. Trials remain in progress for certain therapeutic targets in ovarian cancer, including tissue factor (tisotumab vedotin, NCT03657043) and mesothelin (anetumab ravtansine, NCT03587311).

Mirvetuximab soravtansine

Folate receptor-alpha (FRα) is a cell surface protein frequently overexpressed in EOC in up to 81.5% of ovarian cancer tumours[99]. Mirvetuximab soravtansine targets this receptor and is linked to a maytansinoid DM4, which is a tubulin targeting agent. In November 2022, the US Food and Drug Administration approved Mirvetuximab soravtansine, a novel antibody-drug conjugate, for platinum-resistant EOC with FRα-positive disease based on phase III SORAYA study[100]. This study included 366 patients expressing FRα who received either mirvetuximab soravtansine or physician's choice chemotherapy[100]. There was no PFS difference seen in either the overall population or FRα-high cohorts; however, the secondary outcomes (ORR, CA125 response, patient-reported outcomes) demonstrated significant improvement in Frα-high subgroups. The ORR was 24% compared to 10%, CA125 response was 53% compared to 25% and patient-reported outcome was superior with mirvetuximab soravtansine with 27% compared to 13%. There was no statistically significant difference in median OS in the overall population or in FRα-high subgroups. It also demonstrated a more manageable safety profile than chemotherapy, mainly resulting in low-grade, reversible ocular and gastrointestinal issues manageable with supportive interventions. Overall, there were fewer treatment-related grade 3 or higher adverse events (25.1% *vs.* 44%) and fewer treatment discontinuations (4.5% *vs.* 8.3%).

FRα as a therapeutic target has shown promise with reasonable adverse event rates, and it may be worthwhile looking for ways to increase the sensitivity of EOC cells to these molecules in future studies.

NaPi2b targeting ADCs

NaPi2b is a cell surface sodium-dependent phosphate transporter and is overexpressed in select cancers including ovarian cancer. Two ADC molecules, lifastuzumab vedotin and upifitamab rilsodotin, have demonstrated modest efficacy in early-phase trials[101,102] and several other studies are underway (NCT03319628, NCT04907968, NCT05329545).

Epacadostat

Another investigational agent, Epacadostat, is a potential therapeutic in a number of tumour types, including ovarian cancer. Epacadostat is an Indoleamine 2,3-dioxygenase-1 (IDO1) inhibitor. IDO1 is a key regulator of immune tolerance in ovarian cancer, facilitating the breakdown of tryptophan and its mechanism, resulting in increased Tregs, myeloid-derived suppressor cells (MDSCs) with decreased tumour infiltration lymphocytes, and NK cells with upregulation of PD-1 in cytotoxic T cells overall leading to an immunosuppressive effect on TME. Overexpression of IDO1 has been associated with worse prognosis in

various cancers,c and up to 56.7% of ovarian cancer has been identified to exhibit high IDO1 expression[103]. With evidence of preclinical studies demonstrating synergistic effects of epacadostat and immune checkpoint blockade, many studies have been conducted in combination with ICI. So far, results have been largely disappointing, with only moderate efficacy seen in the melanoma and renal cell carcinoma cohort in phase III study[104]. A phase II basket study examined the safety of epacadostat with pembrolizumab (NCT02178722) in 44 participants, including 37 with recurrent EOC. Unfortunately, a modest ORR of 8.1% was seen in this cohort. A further basket trial of epacadostat with nivolumab in solid tumours demonstrated a modest ORR of 14% and a disease control rate of 31% in a pre-treated ovarian cancer cohort of 29 patients[105]. Currently, there is a phase I trial (NCT02042430) examining the safety and tolerability of epacadostat prior to cytoreductive surgery for newly diagnosed EOC.

Adavosertib

Adavosertib (AZD1775) is a Wee1 nuclear kinase inhibitor that has emerged as a potential compound able to regulate G2-M transition in the cell cycle and sensitise *TP53*-mutant cells to chemotherapy. Recent phase II trials in platinum-resistant or platinum-refractory settings demonstrated that when adavosertib was combined with gemcitabine, PFS was longer in the combination arm (median PFS 4.6 *vs.* 3 months)[106]. Another single-arm phase II study examining adavosertib with carboplatin demonstrated an ORR of 38% with a median PFS of 5.6 months[107]. Neither study demonstrated safety or toxicity issues, and side effects were reported to be manageable. Adavosertib is currently being examined with olaparib (NCT03579316) in recurrent EOC.

Gemogenovatucel-T

Gemogenovatucel-T (Vigil) is a novel autologous tumour cell immunotherapy with multiple functions, including granulocyte-macrophage colony-stimulating factor (*GMCSF)* gene activation and TGF-ß1 and TGF-ß2 suppression through bi-functional short-hairpin RNA construct targeted to furin leading to generate a systemic immune response. Gemogenovatucel-T has been shown efficacy in phase IIb trial with a recurrence-free survival of 11.5 months (95% CI: 7.5-NR) compared to 8.4 months (95% CI: 7.9-15.5) in placebo arm with significant benefit in *BRCA*-wt patients with HR 0.69 (90% CI 0.44-1.07, one-sided P = 0.078)[108]. Further post-hoc subgroup analysis demonstrated recurrent-free survival and OS benefit in *BRCA*-wt, homologous recombination proficient (HRP) patient population, an effect demonstrated out to up to 3 years[109,110]. It remains unclear as to why this drug was particularly efficacious in the *BRCA*-wt population, but ongoing biomarker studies in EOC patients may reveal further answers in the future.

Gemogenovatucel-T and durvalumab combination were investigated in 5 *BRCA*-wild-type recurrent/refractory EOC in a pilot basket study alongside triple-negative breast cancer patients[111]. Median PFS was 7.1 months with median OS not reached with greater benefit in PD-L1 expressing tumours (n = 8, HR 0.304, 95%CI: 0.0593-1.56, 1-sided P = 0.4715). There were three grade 3 treatment-related adverse events, all related to durvalumab, therefore demonstrating this combination is well-tolerated and has promising clinical activity in recurrent/refractory EOC cohort warranting further investigation.

Nemvaleukin-alfa

Nemvaleukin-alfa is an engineered cytokine binding selectively to intermediate affinity IL-2 receptors (IL-2R), leading to downstream activation of CD8-positive T-cells and NK cells and limited activation of regulatory T cells. Its preliminary activity was demonstrated through the ARTISTRY-1 study (NCT02799095), a phase I/II basket study that included an ovarian cancer cohort of 14 patients in combination with pembrolizumab (ORR 29%) with side effects most commonly being cytopenias[112]. A four-arm, randomised, phase III study is currently underway (NCT05092360) with the same combination using physician's choice chemotherapy as the main comparator arm.

Nanoparticle-based combination immunotherapy

An active field of therapeutic development currently is in the development of nanotechnology with the development of nanoparticles (NP)[113]. These act as drug delivery vehicles capable of providing localised and targeted therapeutic effects of immunotherapy with minimal toxicity when administered due to their targeted nature[113]. The technology is currently in its early days and multiple trials are being undertaken to evaluate its therapeutic value, particularly in relation to NP-bound chemotherapy[113].

Cancer vaccines

As referenced in Table 1 (NCT0320047), cancer vaccines are currently being investigated in ovarian cancer to 'sensitise' the immune system to cancer antigens with the aim of inducing an immune response. Although there have been a plethora of studies investigating various formulations[114], the clinical evidence remains very much in its infancy in treating ovarian cancer, although one recent phase II study in 10 heavily pre-treated, platinum-resistant EOC combining maveropepimut-S, a DPX-platform-delivered peptide cancer vaccine of the antigen survivin, with pembrolizumab and oral cyclophosphamide met its primary efficacy endpoint with 10% partial response and 20% stable disease for more than 12 weeks[115].

CONCLUSION

Despite many advancements in understanding tumour biology and drug development within the last few decades, EOC remains a challenging disease to treat, with unacceptably high recurrence and mortality rates. Improved understanding of tumour biology and resistance mechanisms to current therapy, as well as the intricate interplay between tumour, TME and immune system, has paved the way for the development of immunotherapy. However, despite initial aspirations of transforming EOC from "cold" into "hot", immunogenic tumours with utilisation of ICI, this has been largely disappointing with modest clinical activity, suggesting that there are inherent resistance mechanisms at play within the EOC microenvironment. This review has attempted to highlight key players involved in the immune system and TME in the context of EOC; however, it is by no means a complete or exhaustive description of the myriad of key components and processes which make up the complex ecosystem.

Combining immunotherapies with other drug classes has shown early promise, but larger randomised trials are required to clearly define the role of ICI therapy in the EOC treatment landscape, if any. Importantly further studies in the mechanistic activity of immunomodulators and their interaction with ICI, TME and immune system to understand the enhanced efficacy of ICI combination therapy will be an important area of research in the future. Novel therapeutic classes such as antibody-drug conjugates and tumour-directed vaccines are of growing interest, and future studies involving biomarker strategies and robust research methods will be needed to determine which EOC patients will ultimately gain the most benefit from specific therapeutic combinations, with the ultimate goal of improving patient survival and quality of life.

DECLARATIONS

Author contributions

Article conceptualisation: Yoon WH, DeFazio A, Kasherman L
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Figure creation: Yoon WH, Kasherman L
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Consent for publication
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Cancer Drug Resistance

Original Article

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Interaction of pregnane X receptor with hypoxia-inducible factor-1 regulates chemoresistance of prostate cancer cells

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Abstract

Aim: The nuclear pregnane X receptor (PXR) is a pivotal regulator of steroid and xenobiotics metabolism and plays an important role in shaping tumor cell responses to chemotherapy. Hypoxia within tumor tissue has multifaceted effects, including multiple drug resistance. The goal of this study was to determine whether PXR contributes to hypoxia-induced drug resistance.

Methods: Metastatic prostate cancer cells were used to study the interaction of PXR and hypoxia-inducible factor-1 (HIF-1 in drug resistance associated with hypoxia. The activities of PXR and HIF-1 were determined by assays for its reporter gene or target gene expression. Co-immunoprecipitation (Co-IP) was used to determine the interaction of PXR and HIF-1. Ablation or inhibition of PXR or HIF-1 was used to determine their roles in hypoxia-induced chemoresistance.

Results: PXR was activated by hypoxia, leading to increased expression of multidrug resistance protein 1 (MDR1). Inhibition of PXR by pharmacological compounds or depletion by shRNAs reduced the hypoxic induction of MDR1 and sensitized prostate cancer cells to chemotherapy under hypoxia. HIF-1 was required for PXR activation under hypoxia. Co-immunoprecipitation results showed that HIF-1 and PXR could physically interact with each other, leading to crosstalk between these two transcription factors.

Conclusion: PXR contributes to hypoxia-induced drug resistance in prostate cancer cells through its interaction with HIF-1.

Keywords: PXR, HIF-1, prostate cancer, chemoresistance, multidrug resistance, MDR1, hypoxia

INTRODUCTION

Hypoxia is a common occurrence in solid tumors as a result of the limited vasculature and the deregulated proliferation of cancer cells[1]. Tumor hypoxia elicits profound changes in cellular behaviors that influence cellular metabolism, genetic stability, angiogenesis, and self-renewal[2,3]. Tumor cells in the hypoxic regions are more resistant to chemotherapy than the cells in normoxic regions[4]. Elucidation of the mechanism involved in hypoxia-induced drug resistance can lead to new approaches to improve the efficacy of chemotherapy.

Hypoxia-induced factors (HIFs), which belong to the basic-helix-loop-helix family of transcription factors, play a central role in cellular and systemic responses to oxygen deficiency[5]. HIF-1, the hypoxia-inducible subunit of heterodimeric HIF-1, has been suggested as a promoter of tumorigenesis. The association between HIF-1 overexpression, treatment failure, and poor prognosis has been extensively reported[6-8]. HIF-1 plays an important role in hypoxia-induced drug resistance and could be an interventional target for drug resistance[9,10]. While the protective effects of HIF-1 against chemotherapy are attributed to its interference with cell cycle arrest and apoptosis and induction of multidrug resistant gene (MDR1) expression[11,12], the molecular mechanisms underlying the HIF-1-mediated chemoresistance remain elusive.

Pregnane X receptor (PXR, NR1I2), a member of the nuclear receptor superfamily, is also known as steroid and xenobiotic receptor. PXR regulates cellular response to xenobiotics through the induction of drug-metabolic enzymes (DME) and transporters. Upon binding with ligands and activation, PXR translocates from the cytoplasm to the nucleus and regulates the transcription of target genes[13]. The significance of PXR in cancer chemoresistance is underlined by its activation by commonly used agents of chemotherapy, and its putative role in the regulation of the expression of DMEs and efflux transporters. We have reported that activation of PXR with agonists led to increased resistance of prostate and breast cancer cells to Taxol, vinblastine and tamoxifen, indicating an important role of PXR in chemoresistance[14,15].

Prostate cancer is one of the most common malignancies affecting men, and chemotherapy is part of the standard of care for metastatic castration-resistant prostate cancer. In this study, we tested the hypothesis that PXR is a determinant of hypoxia-induced drug resistance in prostate cancer. Here, we report that HIF-1 activated PXR under hypoxic conditions, leading to increased expression of MDR1 and increased resistance of prostate cancer cells towards chemotherapy. Interestingly, activated PXR and HIF-1 physically interacted with each other. These results suggest an intricate interaction between PXR and HIF-1 in shaping the hypoxic tumor response to chemotherapy.

METHODS

Materials

Phoenix Ampho and 293T packaging cell lines were purchased from Allele Biotechnology. The human prostate cancer cell lines LNCaP, DU145 and PC-3 were obtained from American Type Culture Collection. PXR (G-1) mouse monoclonal antibodies were purchased from Santa Cruz Biotechnology. HIF-1 rabbit monoclonal antibodies were purchased from Cell Signaling Technology. Myc-tag mouse monoclonal antibodies were purchased from Applied Biological Materials Inc. The real-time polymerase chain reaction

(PCR) reagents, dual luciferase reporter assay system, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based cell viability assay kits were obtained from Promega Corporation. DNAfectin liposome transfection reagents were purchased from Applied Biological Materials Inc. Apoptosis Assay kit was purchased from R & D Systems. Rifampicin was purchased from Tocris Bioscience. Ketoconazole, puromycin, and doxorubicin were obtained from Sigma. Taxol was obtained from Enzo Life Science. Doxycycline and cobalt chloride were obtained from Fisher Scientific. pGL3-HRE and pDNA3-HIF-1 were purchased from Addgene Company. PCDH (pCDH vector, purchased from System Bioscience.)-myc-HIF-1, pGL3-PXRE, and pBabe-PXR were constructed in our lab. The pGIPZ lentiviral shRNA constructs against HIF-1 and pTRIPZ lentiviral shRNA constructs against human PXR were purchased from Open Biosystem.

Cell culture and stable transfection

Prostate cancer cells were grown in Roswell Park Memorial Institute (RPMI) 1,640 medium with 10% Fetal bovine serum (FBS) and 1% antibiotics under an atmosphere containing 5% CO_2 at 37 °C in a humidified incubator. Phoenix Ampho cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% antibiotics. Regarding HIF-1 pGIPZ lentiviral shRNA and PXR pTRIPZ lentiviral shRNA viral preparations, 293T cells were transfected with the shRNA constructs in the presence of packaging plasmids (System Bioscience), according to the manufacturer's protocols. LNCaP cells with HIF-1 GIPZ lentiviral shRNA expression were marked by green florescent protein (GFP) and selected by fluorescence-activated cell sorting (FACS). LNCaP cells with PXR pTRIPZ lentiviral shRNA expression were regulated by a Tet-On system and marked by red fluorescent protein (RFP) in the presence of doxycycline treatment. For cell culture under hypoxia, hypoxia was induced by GasPak EZ Anaerobe Pouch System (Becton Dickinson and Company).

Immunocytochemistry

LNCaP cells were seeded into six-well plates containing cover glass and incubated under normoxic or hypoxic conditions for 24 h. Cells were fixed with 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 1 min, and blocked with 1% bovine serum albumin in PBS for 30 min. Cells were then incubated overnight at 4 °C with mouse monoclonal anti-PXR primary antibody at 1:100 dilution and sequentially incubated with Alexa Fluor 488 goat anti-mouse secondary antibody for 1 h at room temperature. The slides were then washed, mounted in Prolong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Molecular Probe), and visualized with a BX41 system microscope (Olympus).

Cell viability assays

Cell viability and proliferation were MTS assay. Briefly, 5×10^4 cells were seeded onto 96-well plates with 200 μL medium per well and incubated in standard cell culture conditions. After 24 to 72 h of incubation, 20 μL MTS was added to each well and incubated at 37 °C for 2 h. The OD values at 490 nm wavelength were then obtained.

Anticancer drugs

To determine the sensitivity of tumor cell lines to anticancer drugs, the viabilities of cells after treatments were determined by MTS viability assay according to the manufacturer's instructions. Specifically, under normoxic or hypoxic conditions, 1×10^4 cells were incubated with different doses of anticancer drugs for 48 h. Hypoxia was induced by GasPak EZ Anaerobe Pouch System. After the treatments, the cells were incubated with the MTS solutions and the absorbances were measured at 490 nm.

Induction of apoptosis in tumor cells by Taxol was determined by two-color analysis using propidium iodide (PI) and FITC-conjugated annexin V based on the manufacturer's instructions. After incubations

with or without Taxol for 48 h under normoxic or hypoxic conditions, the cells were stained with PI and FITC-conjugated annexin V and analyzed with a flow cytometer.

Real-time PCR

Total RNA was isolated from the cultured cells using the PerfectPure RNA Purification System (5 Prime). The quantity and quality of different RNA samples were determined by the 260:280 nm absorbance ratios. The RNA samples were reverse transcribed with random hexanucleotide primers and ProtoScript Moloney Murine Leukemia Virus (M-MuLV) Taq RT-PCR kit (New England Biolabs). The cDNA samples were used by real-time PCR using an Applied Biosystems 7,500 real-time PCR system. Sequences for the primers are listed in Table 1. The relative expression was calculated using the ΔΔCt method and normalized to β-actin RNA levels.

Western blot

Cells were scraped off the plate and directly lysed with 2 × Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis sample buffer. The lysate was boiled, sonicated, and centrifuged. Supernatants were loaded into a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride PVDF membrane. The membrane was blocked for 1 hour and incubated with primary antibody overnight at 4 °C. After washing with Tris-buffered saline with 0.1% Tween® 20 detergent (TBS-T) three times for a total of 15 min, the membrane was incubated with fluorescently-labeled secondary antibody for 1 hour. After washing three times, the blots were scanned by an Odyssey infrared imaging system (LI-COR Biosciences).

Luciferase assay

Cells were seeded onto 12-well plates and transfected with luciferase reporter constructs pGL3-PXRE or vector controls in the presence of the control Renilla luciferase construct. The pGL3-PXRE was the PXR reporter construct containing two PXR-responsive fragments in *CYP3A4* promoters as previously described[14,15]. Cells were harvested with the passive lysis buffer 48 h after transfection, and luciferase activity was determined by dual luciferase reporter assay system according to the manufacturer's protocol (Promega).

Co-immunoprecipitation assay

The 293T cells were transfected with pBabe-PXR or co-transfected with PCDH-myc-HIF-1 and pBabe-PXR. The transfected cells were lysed in a non-denaturing Radioimmunoprecipitation assay (RIPA) buffer (pH of 8.0) containing 20 mM Tris-HCl, 137 mM NaCl, 2 mM Ethylenediaminetetraacetic acid (EDTA), and 1% NP-40 containing protease inhibitors. The cellular lysates were agitated slowly at 4 °C for 20 min and clarified by centrifugation at 12,000 g at 4 °C for 20 min. To immunoprecipitate PXR, 1 mg supernatants were incubated overnight at 4 °C with 4 g mouse monoclonal anti-PXR antibody, followed by mixing of the cell lysates with 100 L protein G-coupled sepharose beads and agitation for 4 h at 4 °C. After washing 3 times with non-denaturing RIPA buffer, the beads were collected by centrifuging at 5,000 g at 4 °C for 1 min. The bound proteins were solubilized with SDS sample buffer and subjected to SDS-PAGE for Western Blot analyses.

Statistics

Two-tailed Student's t-tests with a significance level of 0.05 were used to analyze the differences between the two experimental groups. All results were expressed as mean ± SD.

RESULTS

PXR is activated by hypoxia and stimulates MDR1 expression

Previously, we reported that PXR activation was responsible for increased resistance towards chemotherapy in prostate and breast cancers[14,15]. As a receptor for xenobiotics, PXR activation requires its translocation from the cytosol to the nucleus to regulate the expression of its target genes[16]. First, we performed immunofluorescence analysis and found that PXR was localized to the nucleus when cultured under hypoxic conditions but was mostly localized to the cytosol under normoxic conditions [Figure 1A]. Next, we determined whether hypoxia can affect the transcriptional activity of PXR using luciferase reporter gene assay. As shown in Figure 1B, compared to normoxic conditions, the activity of PXR was significantly increased in LNCaP cells when cultured under hypoxic conditions. The results indicated that PXR was activated and translocated from the cytosol to the nucleus under hypoxic conditions in LNCaP cells.

PXR activation can stimulate the expression of DMEs and efflux transporters, such as MDR1(*ABCB1*)[14]. To determine whether PXR activation by hypoxia can lead to altered expression of MDR1, we knocked down PXR expression in LNCaP cells using three independent shRNAs in LNCaP. As shown in Figure 1C and D, the shRNAs effectively suppressed mRNA expression and decreased the activity of PXR under hypoxic conditions. We then evaluated mRNA levels of MDR1 in LNCaP cells with PXR knockdown. In vector control cells, MDR1 expression had a 6.7-fold increase when cultured under hypoxic conditions compared to normoxic conditions [Figure 1E]. However, the hypoxia-stimulated 6.7-fold increase in MDR1 expression was reduced to only 2.7 to 3.8-fold in cells with PXR knocked down [Figure 1E], suggesting that PXR was at least partially responsible for MDR1 induction by hypoxia.

PXR activation contributes to hypoxia-induced chemoresistance

To model the effects of hypoxia on the drug sensitivity of prostate cancer cells, we plated out and cultured LNCaP cells in media with 10% FBS, subjected them to normoxic or hypoxic conditions, and determined their survival by MTS assays after treatment with Taxol for 48 h. LNCaP cells were more resistant to Taxol under hypoxic conditions than those under normoxic conditions [Figure 2A]. Next, we determined whether PXR activation is responsible for chemotherapy resistance under hypoxic conditions. We evaluated whether hypoxia-induced chemoresistance can be reduced by ketoconazole, an antagonist of PXR which suppresses PXR activation via disruption of the interaction of PXR with the co-activator steroid receptor co-activator-1[17]. Results showed that treatment with ketoconazole restored the sensitivity of LNCaP cells to Taxol in hypoxic conditions [Figure 2B]. We further evaluated the effects of PXR knockdown on hypoxia-induced drug resistance. After treatment with Taxol in hypoxic conditions, the cell viability of these PXR knockdown cells was evaluated by MTS assays. Consistent with the results of antagonist experiments, knockdown of PXR by shRNAs restored the sensitivity to Taxol in one group (sh514 group) [Figure 2C]. However, in the other two groups (sh516 and sh546 groups), PXR knockdown reduced, but did not abolish, the hypoxia-induced drug resistance [Figure 2C].

Since cell viability can be multifactorial, we evaluated the apoptosis induced by Taxol via Annexin V staining in LNCaP cell lines under normoxic and hypoxic conditions. As shown in Figure 2D, the percentage of Taxol-induced apoptosis was reduced from 27% under normoxic conditions to 9% under hypoxic conditions. Taxol-induced apoptosis was also evaluated in PXR knockdown cells by Annexin V staining. Compared with the vector control, shRNA knockdown of PXR significantly increased the percentage of apoptotic cells under hypoxic conditions [Figure 2E]. These results suggest that PXR contributes to the increased resistance of hypoxic tumor cells to Taxol-induced apoptosis.

HIF-1 is required for PXR activation under hypoxic conditions

HIF-1 is a master regulator of adaptive responses to hypoxia and has been demonstrated to be related to hypoxia-induced chemoresistance. To test whether HIF-1 induction is required for PXR activation under hypoxic conditions, we determined the activities of PXR in LNCaP cells treated with chrysin, a natural

Table 1. Primers used in real-time PCR

Gene	Forward primer	Reverse primer
HIF-1	GCCACCACTACCACTGCCACC	GCTCTGTTTGGTGAGGCTGTCCG
HIF-2	AAGCATCCCTGCCACCGTGC	CAACGGCGCTGCTCCCAAGA
HK1	TGAAGGGCGGATCACCCCGG	CTGCTCGGCCAAGCGGTAGG
HK2	CCACGCGCCTGTGAATCGGAG	CTCATCAGAGAGGCGCATGTGGT
PFKL	AAGTGATGGGCCGGCACTGC	GCCGCACTGACTGGTTCCCC
PKM2	CTACCGGCCCGTTGCTGTGG	TTGCTGCCCAAGGAGCCACC
PXR	GCAGGAGCAATTCGCCATT	TCGGTGAGCATAGCCATGATC
C-met	CCAATGGCCTGCAGCCGTGA	TGCCGCTCCTGTCCTGAGCA
EPO1	GCTCACTCGGCACCCTGCAAA	TGCCACCAAGGGAGTGCCCA
IGFβ	GAGGGTGGAGCCTCCTGGGG	GCCTCCGAGCACCCTCCTGA
LDHA	TGCCACCTCTGACGCACCAC	GGCATGTTCAGTGAAGGAGCCAGG
BNip3	GGGGTGGCCACGTCACTTGT	AGTAGGTGCCTTCAGCAGAAAACTG
NIX	CCCAGATTTGTGTTGAACGA	ACGGGAACTTGTTGCACTTT
β-actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
MDR1	GGTTCAGGTGGCTCTGGATA	TGACTCCATCATCGAAACCA
CYP3A4	TGGCACCGTAAGTGGAGCCTGA	TGCAGTCCATTGGATGAAGCCCA

flavonoid that reduces the stability and inhibits protein synthesis of HIF-1[18]. As shown in Figure 3A, chrysin suppressed the induction of HIF-1 under hypoxic conditions in a dose-dependent manner. Concurrently, the activity of PXR was significantly reduced [Figure 3B].

We also knocked down HIF-1 expression in LNCaP cells with three different shRNAs against HIF-1. The induction of HIF-1 under hypoxic conditions was significantly suppressed in shRNA-expressed cells [Figure 3C]. The activity of PXR in HIF-1 knockdown cell lines was examined, and the results shown in Figure 3D suggest that PXR activity was significantly decreased in cells with HIF-1 knockdown compared with the control cell line.

Activation of PXR by HIF-1 and their interactions

Since the PXR activation by hypoxia was compromised by HIF-1 inhibition by chrysin or knockdown by shRNAs, we determined whether HIF-1 can directly activate PXR. We co-transfected 293T cells with pDNA3-HIF-1 and pGPL2-PXRE and determined the effects of HIF-1 on PXR activities. The results shown in Figure 4A indicate that HIF-1 significantly increased the transcriptional activity of PXR. We also examined the changes in PXR activity in 293T cells following the induction of HIF-1 with $CoCl_2$, a hypoxia-mimicking agent that stabilizes HIF-1[19], and demonstrated that $CoCl_2$ also significantly increased the activity of PXR [Figure 4B].

The finding that PXR could be activated by HIF-1 led us to investigate whether there was direct interaction between these two transcriptional factors. The lysate of 293T cells co-transfected with PCDH-myc-HIF1 and pBabe-PXR was subjected to immunoprecipitation with anti-PXR antibody and subsequently to Western blot with anti-HIF-1, anti-myc tag, and anti-PXR antibody. Two specific bands with molecular weights of about 120 KDa and 110 KDa were detected [Figure 4C], indicating a protein-protein interaction between ectopically expressed HIF-1 and PXR under normoxic conditions.

We further confirmed the interaction of PXR with endogenous HIF-1. After transfection with pBabe-PXR, 293T cells were cultured under hypoxic conditions for 24 h, and co-immunoprecipitation was performed as described in the Methods. We found that PXR bound to endogenous HIF-1 (120KDa) isoform under

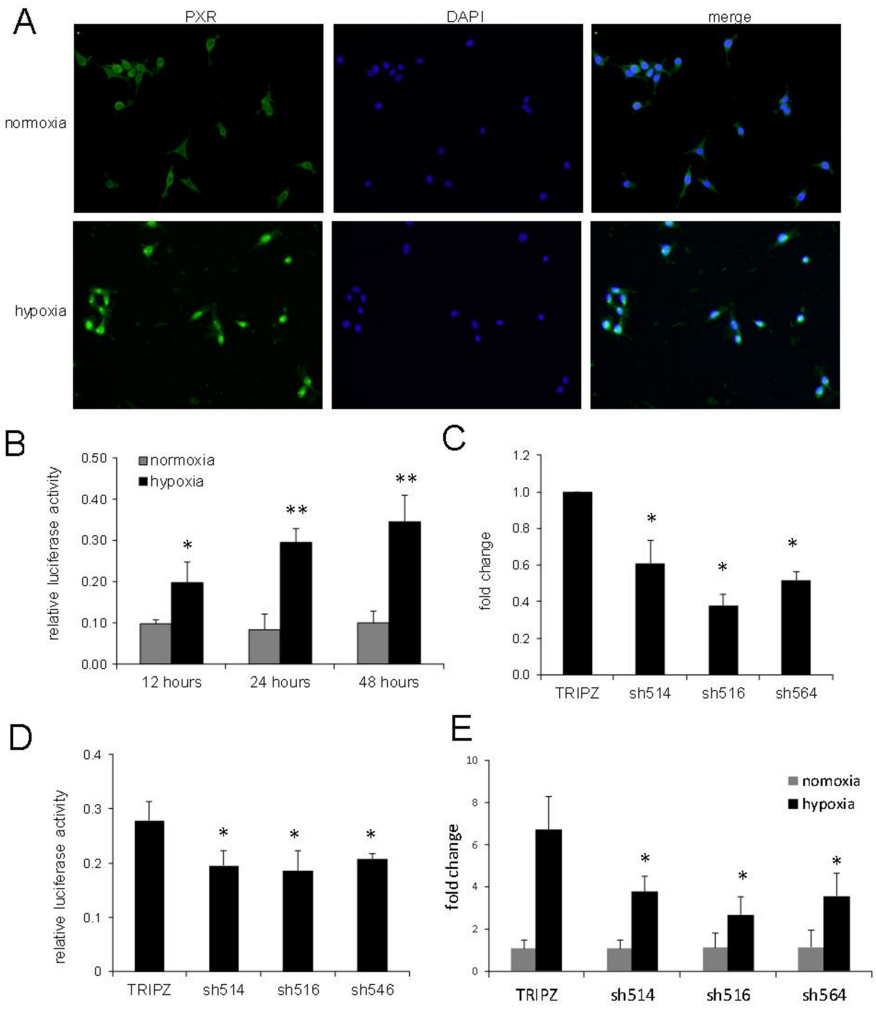

Figure 1. Activation of PXR by hypoxia. (A) Increased nuclear localization of PXR under hypoxia. LNCaP cells cultured under hypoxic or normoxic conditions for 24 h were processed for evaluation of PXR with mouse anti-PXR antibody and DAPI nuclear staining; (B) The activities of PXR in LNCaP cells cultured under normoxic and hypoxic conditions for the indicated durations, as measured by luciferase gene reporter assays; (C) The mRNA expression levels of PXR in LNCaP cell lines with stable expression shRNAs against PXR, as evaluated with real-time PCR; (D) PXR activities in LNCaP cells with stable expression shRNAs against PXR in hypoxia, as determined by luciferase assays; (E) Effects of hypoxia on mRNA expression of MDR1 in LNCaP cells with PXR expression knocked down, normalized with the values obtained from normoxic conditions. The results shown were from at least three independent experiments. Error bars represent standard deviation. *$P < 0.05$; **$P < 0.01$, when compared to normoxic controls (B) or vector TRIPZ controls (C, D, E). MDR1: multidrug resistance protein 1; PCR: polymerase chain reaction; PXR: pregnane X receptor. DAPI: 4′,6-diamidino-2-phenylindole; PCR: polymerase chain reaction.

hypoxia conditions [Figure 4D]. These results suggest a protein-protein interaction between PXR and HIF-1 under hypoxic conditions.

DISCUSSION

PXR has been reported to interact with other transcriptional factors including CAR, FXR, NF-B, and PPAR, suggesting its potential role in various non-canonical functions in addition to its role in metabolism of xenobiotics[20-22]. In the present study, we described the interaction and crosstalk between the nuclear receptor PXR and HIF-1, an important transcriptional factor that shapes cellular responses to changes in the availability of oxygen. Several lines of evidence suggest that the activation of PXR under hypoxic conditions

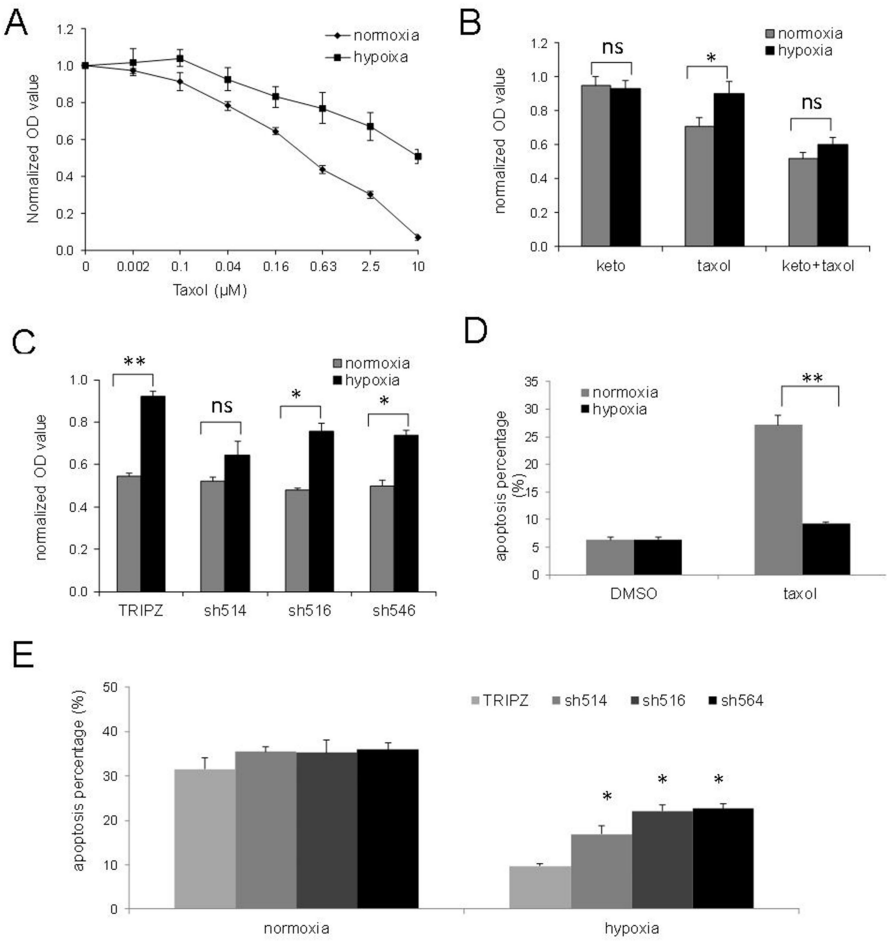

Figure 2. PXR's role in hypoxia-induced chemoresistance. (A) Hypoxia increased resistance of LNCaP cells to Taxol at different dosages; (B) Effects of ketoconazole (10 M) on the sensitivity of LNCaP cells to Taxol (1 μM) under normoxic or hypoxic conditions; (C) Sensitivity of LNCaP cells with PXR knockdown to Taxol (1 M); (D) Hypoxia reduced apoptosis induced by Taxol; (E) Sensitization of hypoxic LNCaP cells toward Taxol-induced apoptosis. LNCaP cells with PXR knockdown treated with Taxol 1 μM were incubated in hypoxic condition for 48 h, and the percentage of apoptosis was determined by PI and annexin V staining followed by flow cytometry assay. The results shown were from at least three independent experiments. Error bars represent standard deviation. *$P < 0.05$. **$P < 0.01$. ns, not significant. PI: propidium iodide; PXR: pregnane X receptor.

was attributed to HIF-1: (1) forced or induced expression of HIF-1 increased PXR activity; (2) hypoxia-induced translocation of PXR from the cytoplasm to the nucleus; (3) HIF-1 inhibition by pharmacologic inhibition or shRNA knockdown abolished the activation of PXR under hypoxic conditions; (4) Co-IP analysis revealed a direct interaction between HIF-1 and PXR. These results strongly suggest that PXR activation by HIF-1 is one of the critical mechanisms underlying hypoxia-induced chemoresistance in prostate cancer cells.

Hypoxia-induced drug resistance poses challenges to effective cancer chemotherapy. Here, we confirmed that hypoxia caused drug resistance in prostate cancer cells. PXR is usually activated in response to xenobiotics, as well as many therapeutics, and plays a potential role in drug resistance during cancer treatment. In our previous study, we found that PXR was expressed in prostate cancer, and its activation led to the expression of MDR1 and *CYP3A4* and increased resistance to chemotherapeutics[14,15]. In this study, we found that hypoxia increased PXR activity and nuclear translocation of PXR in LNCaP cells. PXR

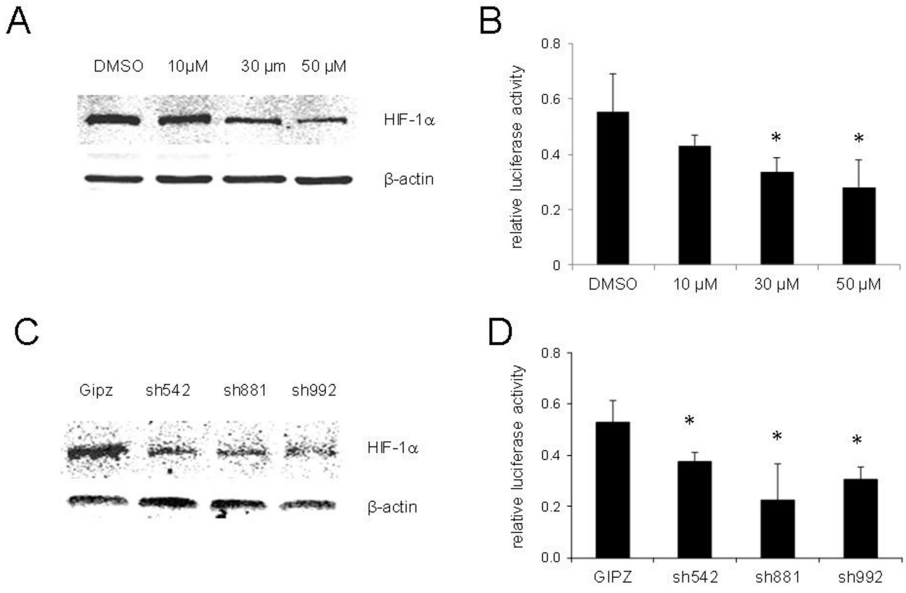

Figure 3. Inhibition or knockdown of HIF-1 suppressed the activity of PXR in hypoxia of LNCaP cells. (A) Expression of HIF-1 in LNCaP cells treatment with chrysin at the indicated concentrations and cultured under hypoxic conditions for 24 h. HIF-1 was detected by Western blot; (B) PXR activity of LNCaP cells treatment with chrysin at the indicated concentrations and cultured under hypoxic conditions for 24 h. PXR activity was determined by luciferase assay; (C) Expression of HIF-1 in LNCaP cells with stable expression shRNA against HIF-1 under hypoxic conditions. HIF-1 was detected by Western blot; (D) PXR activity of LNCaP cell lines with stable expression shRNA against HIF-1 under hypoxic conditions. The fold change was hypoxia/normoxia. The results shown are from three independent experiments. Error bars represent standard deviation. *$P < 0.05$. HIF-1: hypoxia-inducible factor-1; PXR: pregnane X receptor.

knockdown with shRNA or inhibition of PXR activation with the antagonist ketoconazole sensitized tumor cells to Taxol under hypoxic conditions. These results suggest a critical role of PXR in hypoxia-induced chemoresistance in prostate cancer.

While hypoxia-induced drug resistance can be multifactorial, efflux transporters such as MDR1 can contribute to tumor cell resistance to chemotherapy. MDR1 is an ATP-dependent efflux pump with broad substrate specificity. By maintaining reduced intracellular concentrations of antitumor drugs, MDR1 confers cancer cells with multidrug resistance phenotype. Consistent with a previous study[11], MDR1 expression was induced at the transcriptional level under hypoxic conditions. The induction of MDR1 by hypoxia was compromised by either PXR or HIF-1 knockdown, suggesting that PXR activation by HIF-1 was responsible, at least partially, for the induction of MDR1.

The stability and activity of HIF-1 are tightly regulated, primarily through an oxygen-dependent pathway. Under normoxic conditions, HIF-1α is hydroxylated at conserved proline residues by prolyl hydroxylases and ubiquitinated by a pVHL-containing E3 ubiquitin ligase, resulting in rapid degradation by proteasomes. Under hypoxic conditions, HIF-1α is stabilized and regulates the expression of target genes[23]. HIF-1 also has been reported to be regulated by cytokines, growth factors, environmental stimulators, and other signaling molecules in an oxygen-independent manner[24]. We found that PXR binds to ectopically expressed HIF-1 under normoxic conditions and endogenous HIF-1 under hypoxic conditions. While the isoform (110 KDa) of HIF-1 was found to interact with PXR under normoxic conditions, it is the putative isoform HIF-1 (120 KDa) that interacts with PXR under hypoxic conditions. There are isoforms of HIF-1 that can suppress the activity and down-regulate mRNA expression of HIF-1[25,26]. Further studies are

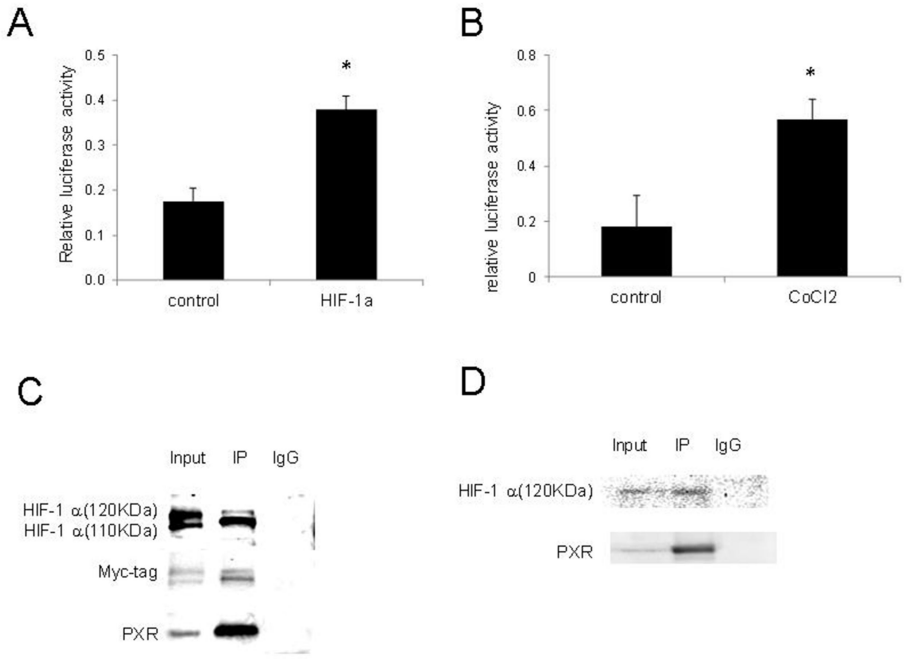

Figure 4. Activation of PXR by HIF-1 and their interactions. (A) HIF-1 increased PXR activity. 293T cells transiently co-transfected with pGL3-PXRE, PCDH-HIF-1, and pGL3-Renilla. PXR activity was determined by luciferase assay 48 h after transfection. The results shown are from three independent experiments. Error bars represent standard deviation. *Indicates significance ($P < 0.05$); (B) $CoCl_2$ increased PXR activity. 293T cells transiently co-transfected with pGL3-PXR and pGL3-Renilla and incubated with $CoCl_2$ 100 μM for 24 h. PXR activity was measured by luciferase assay; (C) Co-immunoprecipitation of overexpressed PXR and HIF-1. IP was performed with mouse anti-PXR, followed by immunoblotting with rabbit anti-HIF-1, rabbit anti-myc-tag, and mouse anti-PXR; (D) Co-IP between PXR with endogenous HIF-1 under hypoxic conditions. IP was performed with mouse anti-PXR, followed by immunoblotting with rabbit anti-HIF-1 and mouse anti-PXR. Co-IP: Co-immunoprecipitation; HIF-1: hypoxia-inducible factor-1; PCDH: pCDH vector; PXR: pregnane X receptor; pGL3-PXRE: PXR responsive element in PGL3 vector.

needed to determine whether and how PXR regulates the activities of HIF-1 during the tumor hypoxia response outside hypoxia-associated drug resistance.

In summary, our study identified a novel interaction between nuclear receptor PXR and transcriptional factor HIF-1. Under hypoxic conditions, HIF-1 increased the activity of PXR, leading to increased expression of MDR1 and increased resistance of prostate cancer cells to chemotherapy. PXR physically interacted with HIF-1 under hypoxic conditions. Our study highlights the pleiotropic effects of PXR on prostate cancer cells regarding the tumor hypoxia response and resistance to chemotherapy.

DECLARATIONS

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Authors' contributions

Made substantial contributions to the conception and design of the study, performed data analysis, and interpreted the data: Wang J, Nie D

Performed data acquisition: Wang J

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Both authors declared that there are no conflicts of interest.

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Cancer Drug Resistance

Review

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Recent advances in access to overcome cancer drug resistance by nanocarrier drug delivery system

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Abstract

Cancer is currently one of the most intractable diseases causing human death. Although the prognosis of tumor patients has been improved to a certain extent through various modern treatment methods, multidrug resistance (MDR) of tumor cells is still a major problem leading to clinical treatment failure. Chemotherapy resistance refers to the resistance of tumor cells and/or tissues to a drug, usually inherent or developed during treatment. Therefore, an urgent need to research the ideal drug delivery system to overcome the shortcoming of traditional chemotherapy. The rapid development of nanotechnology has brought us new enlightenments to solve this problem. The novel nanocarrier provides a considerably effective treatment to overcome the limitations of chemotherapy or other drugs resulting from systemic side effects such as resistance, high toxicity, lack of targeting, and off-target. Herein, we introduce several tumor MDR mechanisms and discuss novel nanoparticle technology applied to surmount cancer drug resistance. Nanomaterials contain liposomes, polymer conjugates, micelles, dendrimers, carbon-based, metal nanoparticles, and nucleotides which can be used to deliver chemotherapeutic

drugs, photosensitizers, and small interfering RNA (siRNA). This review aims to elucidate the advantages of nanomedicine in overcoming cancer drug resistance and discuss the latest developments.

Keywords: Cancer, nanomedicine, nanomaterials, drug delivery, multidrug resistance

INTRODUCTION

Physiologically, the formation of tumor cells is an uncontrolled and unnecessary growth of cells that require a large number of nutrients. According to the world health organization (WHO) report, cancer is the first or second leading cause of death in 112 out of 183 countries. One of the most common causes of death in cancer patients is the development of multidrug resistance (MDR)[1,2]. The tendency of tumor cell drug resistance and its mortality rate is increasing yearly[3]. Based on the development of the current medical level, chemotherapy is a conventional treatment for tumors. Although chemotherapy has made a great success, due to the lack of targeting and limited bioavailability, its efficacy is still facing severe challenges in clinical practice. More importantly, tumor cells will generate MDR, which can enhance the response threshold of other cytotoxic drugs after long-term administration of chemotherapy drugs[4,5]. Novel molecular targeted therapy and immunotherapy have become methods to overcome the lack of specificity of traditional chemotherapy drugs[6]. However, due to the development of drug resistance, cancer cells still can evade the cytotoxicity of newer molecular-targeted therapeutic drugs. Therefore, resistance to drugs is a common cause of death in cancer patients treated with conventional chemotherapy or novel targeted drugs. MDR is a highly heterogeneous disease state. When a tumor cell becomes resistant to a single anticancer drug, it may become cross-resistant to a range of drugs with different structures or mechanisms. It is one of the key factors for recurrence and metastasis of tumor patients[7].

The decrease of drug accumulation in cells is the common cause of drug resistance in tumor cells. At present, it is generally believed that tumor cells develop tolerance to drug toxicity in two ways: one is to prevent drugs from reaching the target site through a series of self-regulation when drugs enter the cell from the outside; the other is to pump drugs into the cytoplasm directly outside the cell so that the concentration of drugs available inside the cell is reduced. Up to now, the mechanism of tumor drug resistance has not been fully understood[8,9]. Cancer resistance is divided into two broad categories: primary resistance, with an early tumor progression, without prior tumor response, and secondary (acquired) resistance, which occurs after initial tumor responses[10,11]. Among them, primary resistance may be mainly related to the lack of target dependence or the presence of other targets. The main mechanisms of acquired resistance include on-target resistance alterations, bypass alterations in the same pathway or connecting pathways, and changes in phenotypic transformation of tumor cells[12,13].

The application of nanomaterials in the medical field is also called nanomedicine. This new treatment method can combine the delivery of drugs and improve the treatment effect while protecting normal tissues from side effects. In this paper, we start with the mechanism of tumor drug resistance and introduce the molecular mechanisms related to tumor drug resistance, such as the ATP-binding cassette (ABC) transporter family and MDR mediated by the enzyme system. Next, the research of a nano-drug delivery system in reversing tumor drug resistance was introduced, including the value of nanocarrier platforms in improving the solubility of chemotherapy drugs, improving drug targeting, multidrug delivery, and others. Finally, this paper expounds on the nano-drug delivery system reversal of tumor resistance to remedy the technical advantages and application prospects.

MECHANISMS OF RESISTANCE IN CANCER

More than half of cancer deaths are due to the generation of drug resistance[2]. The drug resistance of tumor cells is involved in a variety of mechanisms, including the increase of MDR during long-term chemotherapy, DNA repair ability enhancement, genetic mutation, heterogeneous biological metabolism, blocked apoptosis pathway, microenvironment changes, and drug target alteration, among others[14-16]. Any one or more of the above mechanisms will reduce the drug efficacy and increase the difficulty of tumor treatment. Therefore, from the perspective of molecular mechanisms, the study of molecular markers and molecular targets for reversing tumor resistance is the focus of tumor therapy [Figure 1]. Common MDR mechanisms that lead to drug resistance in tumor cells are briefly highlighted in the following sections.

Efflux of drugs mediated by ABC transporter family

The ABC transporter is a transmembrane protein that relies on the energy produced by the hydrolysis of ATP to shuttle the substrate through the channels of the cell membrane[17,18]. It is mainly responsible for regulating the distribution, absorption, and excretion of various compounds. Among the ABC transporters that have been discovered, the primary proteins related to tumor cell drug resistance are p-glycoprotein (P-gp/MDR-1, or ABCB1)[19], multidrug resistance-related protein (MRP-1/ABCC1)[20], breast cancer resistance protein (BCRP/ABCG2)[21]. In follow-up studies, scientists found that these proteins were up-regulated in various MDR cancer cells[22-24]. Among them, the most typical cause of MDR is the overexpression of P-gp. P-gp has a self-protection mechanism, which can efflux most chemotherapeutic drugs out of the cell to reduce intracellular drug accumulation. The substrates of P-gp protein are widespread, including varieties of antitumor drugs, such as paclitaxel, cyclophosphamide, DOX, and emerging molecular targeted drugs[25-28]. In brief, resistance is more likely to develop in P-gp overexpression tumor cells than that in naturally low P-gp expression ones. Huang[29] clarified that the drug released by nanoparticles into the cytoplasm is susceptible to P-gp-mediated drug efflux. Based on the above facts, P-gp-induced drug efflux is considered one of the major reasons for drug resistance.

Tumor microenvironment and the enzyme system

The tumor microenvironment (TME) refers to the environment in the tumor cells, including mesenchymal cells and capillaries, the secretion of extracellular matrix, factors, and so on. TME contains a variety of stromal cells, which are mainly composed of tumor-associated macrophages (TAMs). As a heterogeneous population with plasticity, macrophages in TAMs exhibit two polarization states during maturation and differentiation through two different activation pathways, namely classical activation type (M1) and alternative activation type (M2), among which M2 TAMs are believed to play a key role in tumor resistance and disease progression[30]. In short, TAMs can participate in the generation of drug resistance in tumor cells by releasing cytokines, regulating signaling pathways, intervening in angiogenesis, and interacting with tumor stem cells[31].

Compared with normal tissue, the tumor microenvironment has abnormal physiological and biochemical characteristics, such as low extracellular pH, hypoxia, high intracellular glutathione (GSH) levels, and others. It affects the occurrence and development of tumors, including proliferation, angiogenesis, invasion, migration, drug resistance, and so forth[32-36]. It is reported that Hypoxia-inducible factor-1α (HIF-1α) can significantly increase the expression of ABC transporters (MDR1, MRP1, BCRP) to induce cell resistance[37,38]. In addition, HIF-1α is found overexpressed in breast cancer and enhances the drug resistance of MCF-7 cells to tamoxifen by mediating tumor cell autophagy[39]. GSH is a tripeptide, and glutathione transferase can catalyze glutathione. GSH plays a key role in protecting cells from oxidative stress. Importantly, it not only regulates the pH of the microenvironment in drug-resistant tumor cells, but also plays a key role in regulating cell resistance to drugs. GSH can indirectly enhance drug resistance by inhibiting the RAS-MAPK pathway and activating protein kinase[40,41]. Topoisomerase II can form the Topo

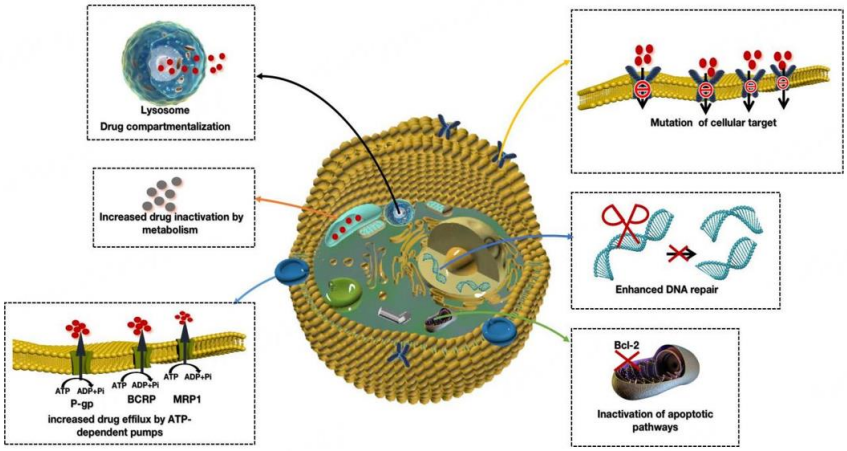

Figure 1. This diagram depicts the expulsion of cells by efflux transporters, including P-gp, BCRP, and MRP-1. Other mechanization includes drug inactivation by increasing the expression of enzymes that metabolize drugs and the development of resistance. Cells can develop resistance by changing the target of a drug, thus disabling the drug. Drug isolation into lysosomes can cause drug inactivation and cause cancer cells to acquire resistance. Drugs inhibit cell apoptosis and produce drug resistance by interfering with the genetic cycle of cells. BCRP: Breast cancer resistance protein; Bcl-2: B-cell leukemia protein 2; MRP: multidrug resistance-related protein; P-gp: p-glycoprotein.

DNA complex with drugs, and thus inhibit the expression of Topo or increase the phosphorylation level, reducing the enzyme content and activity, and leading to the reduction of drug action targets and drug resistance[42]. The Ca^{2+}-dependent protein kinase system is also one of the enzyme systems that promote drug resistance in tumor cells. MAPK pathway is one of the important downstream targets regulated by calmodulin-dependent protein kinase II (CaMKII). CaMKII can regulate the survival and proliferation of MDR cancer cells by director indirectly up-regulating protein kinase C (PKC), extracellular signal-related kinases 1 and 2 (ERK1/2), AKT1, and other signaling pathways. PKC is widely distributed in various tissues and cells, which can mediate signal transduction and regulate gene expression and is related to tumorigenesis and drug resistance[43-45].

In recent studies on some biomarkers overexpressed in the tumor microenvironment, many targeted nanoparticles have been developed for drug delivery. Excitingly, these studies also found that some nanoparticles can competitively bind to drug-resistant cytokines and enzymes, thereby blocking the proliferation of drug-resistant cells.

Transdifferentiation and tumor cell resistance

Under the effect of anticancer drugs, the tumor cell adopts distinct phenotypes to adapt to changes in their environment - This representation is commonly referred to as cell plasticity[46]. This characteristic of transforming cells into tolerant or resistant cell states/phenotypes no longer relies on existing drug targets and pathways, thus escaping drug attacks[47]. The earliest clinical evidence of cell plasticity came from patients with rare non-small cell lung cancer (NSCLC) who showed resistance to epidermal growth factor receptor (EGFR) inhibitors, and some of the patient's tumor tissue type transformed into small cell lung cancer (SCLC). Interestingly, Some patients regained sensitivity to EGFR inhibitors after discontinuation[48]. Notably, there were differences in cell phenotypes after the transdifferentiation of resistant cells. In another case of a patient with non-small cell lung cancer, the drug-resistant cells metastasized into neuroendocrine cells[49]. The most common phenomenon of drug-induced tumor cell plasticity is epithelial-mesenchymal cell transformation (EMT)[50]. In addition, lineage transdifferentiation and proliferation-invasion phenotypic transformation are also manifestations of cell plasticity. EMT is regulated by transcription

factors mainly from SNAIL, TWIST, and ZEB families[51-53]. TGF-β, FGF, EGF, HGF, IGF-1, and members of the Hedgehog, Notch and Wnt signaling pathway - can induce EMT[54-56].

Regulation of cell metabolism in drug-resistant tumor cells

A common feature of tumor cell resistance is as much as possible to reduce glucose consumption by mitochondrial respiration. In many experiments, biological processes related to mitochondria were observed to be activated or enhanced in tumor-resistant cells. In addition, different types of tumors may differ in how they metabolize glucose. Superoxide produced by mitochondrial aerobic respiration can cause oxidative stress in tumor cells[57-59]. Therefore, tumor-resistant cells require a potent antioxidant process in response to superoxide[60,61]. One of its antioxidant pathways is that glutathione peroxidase 4 (GPX4) catalyzes the reduction of glutathione. GPX4 is used to reduce intracellular lipid peroxide to reduce oxidative stress[44]. The other important antioxidant pathway is aldehyde dehydrogenase (ALDH) which protects drug-resistant cells from the toxic effects of reactive oxygen species[62].

Cells spend more than half of their energy on protein synthesis, so tumor-resistant cells must minimize their protein synthesis[63]. Whole-genome studies of different tumors have shown that the proliferation slowdown is deeply related to reduced protein synthesis. Posttranscriptional modification of N6-methyladenine (N6-methyladenosine, m6A) has been found to reduce the translation efficiency of drug-resistant tumor cells in leukemia and melanoma[64].

Regulation of apoptosis in drug-resistant tumor cells

The eventual aim of most anticancer drugs is to promote tumor cell death. Consequently, the destruction of the apoptosis mechanism may give rise to the drug resistance of anticancer drugs. Defects in apoptosis occur when genes that control apoptosis are activated and are thought to complement the activation of proto-oncogenes[65]. Thus, many unregulated oncoproteins, such as MYC, B-cell leukemia protein 2 (Bcl-2), and p53, have a weakened role in triggering apoptosis, which results in the drug resistance of cancer cells[66].

In the apoptotic pathway, the changes of influential factors such as Bcl-2, nuclear factor NF-kappaB (NF-κB), and tumor necrosis factor (TNF) reduce the sensitivity of tumor cells to drugs, and thus inhibit the apoptosis of tumor cells and lead to drug resistance in tumors[67,68]. Among them, the molecular mechanism of the Bcl family inducing apoptosis is one of the few apoptosis pathways targeted by Food and Drug Administration (FDA) approved drugs[69]. Recent studies have identified another anti-apoptotic protein, myeloid cell leukemia sequence 1 (MCL-1), as a compelling reason for adaptive drug resistance in tumor cells treated with a series of targeted therapies (including BRAF inhibition and EGFR inhibition)[67]. Other anti-apoptotic mechanisms may also comprise the trigger of exogenous apoptotic pathways or autophagy[70-72].

Multidrug resistance and autophagy

Autophagy is a highly conserved biological phenomenon, widely existing in eukaryotic cells[73]. It is a way for cells to maintain homeostasis of the intracellular environment and genome stability by self-decomposing damaged organelles and cell components under extreme stress conditions, which is conducive to making cells gain survival advantages under stress and pressure conditions caused by growth or environmental changes[74]. An increasing number of studies indicate that certain drugs can induce autophagy in tumor cells when they are stimulated. On the one hand, autophagy, as the executor of type II programmed death, can initiate the autophagy death mechanism and lead to cell death. On the other hand, autophagy, by protecting the survival of tumor cells, may activate the tolerance of tumor cells to chemotherapy drugs, resulting in reduced sensitivity of tumor cells to chemotherapy drugs and drug resistance[75]. The up-regulation of autophagy function is a significant factor in drug resistance in drug therapy and radiotherapy. It has been

found that the P13K-AKT-mTOR signaling pathway is one of the main pathways mediating protective autophagy, which is mainly involved in the signal transduction of growth factors and hormones[76]. In addition, Autophagy related genes (ATGs), p62, and IL-6 can also mediate autophagy to make some cancer cells acquire drug resistance[77-79].

Enhanced DNA damage repair causes drug resistance in tumor cells

Another possibility for resistance to various anticancer drugs is the enhanced ability of tumor cells to repair DNA damage. Clinical use of cisplatin, DOX, and other chemotherapy drugs can cause DNA damage, which has a significant therapeutic effect. However, repeated DNA damage can induce the abnormal activity of the DNA repair system, leading to increased synthesis of enzyme proteins in tumor cells, weakening the effect of tumor drugs, and resulting in drug resistance[80]. When the cell receives the stimulation after injury, it will activate the DNA damage response (DDR) way to reply. Currently, there are at least five major DNA repair pathways - base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ). They are active at all stages of the cell cycle and enable cells to repair DNA damage[81,82]. It is reported that DNA repair endonuclease xeroderma pigmentosum group F (XPF) and DNA repair protein excision repair cross-complementing 1 (ERCC1), which are involved in the nucleotide excision repair (NER) pathway, are crucial to effectively repair DNA damage induced by cross-linking agents and platinum-based reagents. Studies have shown that the overexpression of XPF and ERCC1 proteins is significantly related to the enhancement of cisplatin resistance of cancer cells[83]. In many tumors, it was also found that the reduction of the DNA mismatch repair pathway can also lead to the increase of drug resistance, and the hypermethylation of the human MutL Homolog 1 (*hMLH1*) gene promoter leads to the decrease of MLH1 protein expression involved in the mismatch repair pathway. 5-fluoro-2-deoxycytidine and decitabine can reverse this hypermethylation and increase the sensitivity of cells to cisplatin[84].

Drug target changes in drug-resistant tumor cells

Drug-resistant tumor cells can also acquire drug resistance through gene mutation in the condition of slow proliferation. Therein, mutations or aberrations of targets are one of the most common mechanisms. From the Cancer Genome Atlas (TCGA) database, people can accurately identify mutation types in thousands of tumors using sequencing methods[85]. Among them, the activating mutations of oncogenes such as EGFR, RAS, RAF, and PI3K are the key driving factors for many cancers[86-89]. With more in-depth research on this mutation generation, many approved drug molecules have been discovered. Many genetic changes lead to the inactivation of tumor suppressor genes in tumor cells, such as PTEN and p16INK4a[90,91]. In addition, these mutated proteins may remain permanently "on" or more activated than wild-type (WT) proteins. These activated protein targets, further mutations of amino acids can also lead to drug resistance through numerous mechanisms. Although the mechanism of drug resistance is a highly concerning clinical problem, if we can grasp the off-target mechanism, it may provide a new choice for follow-up targeted drug therapy.

In conclusion, the important molecular mechanisms related to tumor resistance have been extensively investigated, including the ABC transporter superfamily, enzyme system in vivo, cell apoptosis, autophagy, embryonic stem cells, etc., which can lead to increased drug efflux, enhanced detoxification mechanism, increased DNA repair capacity, elevated metabolism of xenobiotics, and cell death inhibition. Due to the interaction of genes and signal regulatory pathways, these mechanisms can not clearly explain the MDR mechanism of tumors in a certain way. With the further advancement of the research on transcriptome and proteome, the regulatory mechanism of MDR can be explained in a multi-dimensional way, providing a reference for the research on the clinical reversal of tumor resistance.

Drug resistance of tumor cells can occur in any part of tumor cell growth. With the rapid development of modern medical technology, scientists continue to uncover the mystery of drug resistance in tumor cells. Subsequently, a new generation of antitumor drugs with targeting and high efficiency has been developed continuously. However, these chemotherapy drugs still have the shortcomings of cross-resistance, off-target, and damage to normal tissues. Due to the advantages of small particle size, large specific surface area, safety, enhanced permeability and retention effect (EPR effect), and promotion of drug enrichment, nanocrystals have been widely used[92]. For the past few years, it has become a hot topic for scientists to effectively improve the therapeutic effect of antitumor drugs by using nanocarriers to load drugs and external tumor-specific identification of target molecules to establish targeted nano-delivery systems [Figure 2 and Table 1]. In the following, we will introduce some principles of drug resistance in tumor cells, as well as nano-drug delivery strategies targeting these resistance mechanisms.

Nanoparticles, as exogenous substances, will encounter various obstacles at all stages of transportation in vivo. After being injected intravenously into the blood, various proteins in the plasma will be attached to the surface of the nanoparticles and labeled. The reticuloendothelial system and mononuclear macrophages will recognize and remove the labeled nanoparticles and even form vascular embolism or organ infarction. By changing the characteristics of nanoparticles, such as stability, surface charge, surface functional groups, etc., we can improve the circulating ability of nanoparticles in vivo, increase the aggregation and targeting of drugs, and finally reverse the drug resistance of tumors.

NANO DRUG DELIVERY STRATEGY FOR MDR TREATMENT

The nanoparticle delivery platform can regulate drug release and change drug distribution in vivo. Currently, commonly used nanoparticle carriers include liposomes, solid lipids, polymers, mesoporous silica, and inorganic nanoparticles[93,94]. It has been shown that nanoparticles can be designed with different sizes, multiple surface modifications, different shapes, and multiple loading modes[95]. It can improve the solubility and bioavailability of insoluble drugs and enhance the targeting of drugs. At the same time, light, heat, sound, and other stimuli to trigger the release of drugs can reduce the toxic side effects of drugs[96,97]. In the following, we detail the evolution of nanoparticles for overcoming chemoresistant tumors.

The tumors in the clinic are divided into solid tumors and liquid tumors. Solid tumors refer to a class of detectable tangible masses with high clinical incidence[98]. However, non-solid tumors are mainly tumors of the hematopoietic system and lymphatic system, accounting for only 10% of all tumors, and can not be seen in imaging examinations[99]. Unlike organ cancer and other solid tumors, blood cancer (including multiple myeloma, leukemia, and lymphoma) is formed in the bone marrow or the lymphatic system. Existing treatments for blood cancers include chemotherapy, radiation, immunotherapy, and transplantation[100,101]. Chemotherapy is the primary treatment for liquid tumors. However, as anticancer drugs are easy to produce drug resistance, the survival rate of patients is low, so the recurrence rate is high. Therefore, there is an urgent need for a targeted drug delivery system to improve the effectiveness of solid and liquid tumor therapy. After modification and modification of nanomaterials, nanocarriers can be given the ability to actively target and passively target tumor cells (including microenvironment, receptors, ion channels, organelles, etc.), which can increase the chemical drugs enrichment in drug-resistant tumor cells and safety. Meanwhile, nanocarriers themselves or cooperatively delivered various drugs can resist tumor drug resistance through phototherapy-chemotherapy, photothermal-chemotherapy, immunochemotherapy, gene therapy-chemotherapy, and other ways. Different types of nanoparticles have received considerable attention in the therapy of various solid tumors, leading to several successful drug delivery systems entering clinical practice over the years [Table 2]. In the following, we detail the evolution of nanoparticles for overcoming chemoresistant tumors. Table 2 provides a brief selection of currently approved and

Table 1. Characteristics based on partial nano drug delivery systems

Classification	Nanocarriers	Nanocarriers properties	Disadvantage
Organic nanocarriers	Liposomes	(a) Amphiphilic, biocompatible (b) Wide adaptability (c) Targeting potential (d) Ease of modification	(a) Poor stability, easy to be affected by metal radiation, high temperature, pH, and enzymes (b) Low drug loading rate
	Polymeric micelles	(a) Long retention time in the body (b) Suitable carrier for water-insoluble drug (c) Ease of functional modification (d) Biocompatible, self-assembling, biodegradable (e) Special "core-shell" structure, targeting potential	Poor physical stability, resulting in drug leakage and sudden release
	Polymeric nanoparticles	(a) Water-soluble, nontoxic, and biodegradability (b) High drug loading (c) Selective accumulation and retention in tumor tissue (EPR effect) (d) Active targeting and smart response	(a) Easy binding to negatively charged non-specific cells or proteins (b) Low gene transfection efficiency
	DNA/RNA	(a) Easy synthesis and modification (b) Low immunogenicity (c) Excellent specificity and affinity (d) Active targeting and Intelligent drug release	(a) Poor cellular uptake (b) Poor stability
	HSA	(a) Safety, no immunogenicity, good biocompatibility (b) Biodegradable (c) Passive targeting	(a) Large particle size and easy degradation (b) Preparation method is easy to cause increased toxicity (c) Limited sources of HSA
Inorganic nanocarriers	Metal nanoparticles	(a) Biocompatible (b) Easy preparation	(a) Need surface modification (b) Poor biocompatibility
	Non-metallic nanoparticles		(a) Low surface potential (b) Low drug loading (c) Easy aggregation

EPR: Enhanced permeability and retention; HSA: human serum albumin.

investigational nanomaterials in the arena of chemoresistant anticancer therapeutics (approved references are 102 to 109). Table 3 presents the example mentioned in subsequent sections.

INCREASE THE DRUG AGGREGATION IN MDR CELLS-PASSIVE TARGETING

The blood environment is a primary factor affecting drug accumulation in tumor tissue. Studies have shown that the blood environment is the first major obstacle for nano-drug delivery systems[110]. The reticuloendothelial system (RES), which consists of body blood protein, opsonin, liver, and spleen, plays a prime role in the metabolism and clearance of nano drug loading system. Tumor blood vessels are hyperactive, and there is a gap between endothelial cells and the lymphatic system. Vascular leakage is associated with EPR effects[111,112]. Most types of nanocarriers can enhance permeability and retention (EPR) effects[113]. Nanoparticles (NP) can accumulate in tumor tissues and obtain passive targeting by utilizing the unique pathophysiological mechanism of tumor blood vessels to overcome the drug resistance of cancer cells. It was found that the key parameters affecting the effect of NPs EPR were the size and surface of NPs, and particles up to 100 nm in diameter could migrate into tumor tissues[114]. Therefore, nanoparticles can continuously deliver drugs to tumor tissue without increasing the dose of chemotherapy drugs. As nanoparticles usually carry multiple chemotherapeutic drugs or bioactive components with multiple anti-MDR mechanisms, the in-depth development of nanomaterials will gain more benefits for MDR tumor treatment. We further introduce different kinds of nano-drug-loaded particles in the following.

Table 2. Representative examples of nanocarrier - marketed products or products in clinical trials with their indications

Classification	Compound	Indications
Liposome	Liposomal doxorubicin (Doxil, Janssen)	Karposi's sarcoma, ovarian cancer, multiple myeloma
	Liposomal vincristine (Marqibo, Spectrum pharmaceuticals)	Acute lymphoblastic leukemia
	Liposomal irinotecan (Onivyde, Ipsen biopharmaceuticals)	Pancreatic cancer
	Liposomal daunorubicin and cytarabine (Vyxeos, Jazz pharmaceuticals)	Acute myeloid leukemia with myelodysplasia related changes
	Liposomal lurtotecan (OSI-211, phase-II)[102]	Recurrent ovarian cancer, recurrent small-cell lung cancer
	Liposomal paclitaxel (LEP ETU, phase-I/II)[103]	Advanced solid tumors
	Liposomal oxaliplatin (Aroplatin, phase-II)[104]	Advanced colorectal cancer
	Liposomal interleukin-2 (Oncolipin, phase-II)[105]	Immune stimulant for use with a liposomal vaccine against non-small cell lung cancer
Polymeric	Leuprolide acetate and polymer (Eligard, Tolmar)	Prostate cancer
	Pegfilgrastim (Neulasta, Amgen)	Chemotherapy-induced neutropenia
	Dantrolene sodium (Ryanodex, Eagle pharmaceuticals)	Malignant hypothermia
	Pegaspargase (Oncaspar, Baxalta U.S.)	Acute myeloid leukemia
	PEG-PLA/paclitaxel (Genexol-PM)	Breast and lung cancer
	PLGA/goserelin acetate (Zoladex)	Prostate and breast cancer
	PLGA/leuprolide acetate (Lupron depot)	Prostate cancer and endometriosis
	PLGA/triptorelin pamoatea (Trelstar)	Advanced prostate cancer
	PLGA/leuprolide acetate (Eligard)	Advanced prostate cancer
	HPMA-copolymer-doxorubicin (PK1; FEC28069, phase-II)[106]	Lung cancer, breast cancer, and various other cancers
	PEG-camptothecin (Prothecan, phase-II)[107]	Various cancers
	Paclitaxel-poliglumex (CT-2103; Xyotax, phase-III)[108]	Non-small cell lung cancer, ovarian cancer
Protein nanoparticles	Denileukin diftitox (Ontak, Eisai)	Cutaneous T-cell lymphoma
	Albumin-bound paclitaxel (Abraxane, Celgene)	Breast cancer, non-small-cell lung cancer, pancreatic cancer
Metal-based nanoparticles	Colloidal gold nanoparticles coupled to TNF and PEG-thiol (CYT-6091; Cyt-immune Sciences, phase 1)[109]	Solid tumors

PEG: Poly ethylene glycol; PLA: poly lactic acid; PLGA: poly (lactide-co-glycolide); TNF: tumor necrosis factor.

Liposomes

The liposome is one kind of nanocarrier formed by encapsulating drugs in a lipid-like bilayer. Liposomes have excellent biocompatibility due to their similar structure to the plasma membrane of biological cells. Liposome encapsulation of drugs can reduce drug toxicity, improve drug stability and bioavailability, and has gradually been widely applied as a delivery carrier for small molecule drugs and proteins[115]. Doxil®, a lipid-based nanoparticle drug preparation approved by FDA, has been used in the clinical treatment of a variety of malignant tumors (metastatic breast cancer, ovarian cancer, multiple myeloma, etc.). Subsequently, Marqibo®, DaunoXome®, Vyxeos®, Onivyde®, and other liposome nano preparation have also been approved by FDA for tumor treatment[116-119]. The core of the liposome is a hydrophobic region formed by lipophilic groups, and the inside of the lipid bilayer is a hydrophilic region formed by the phospholipid layer, which can co-carry hydrophilic and hydrophobic drugs. Li *et al.*[120] prepared D-α-tocopherol polyethylene glycol-1000 succinate (TPGS) coated liposome (TPGS-liposome) as a drug delivery platform for docetaxel (TPGS-DTX-liposomes). In this drug-loaded nanosystem, liposomes are easily absorbed by cells through endocytosis to increase the cellular uptake of docetaxel. TPGS can prevent Docetaxel (DTX) from being recognized by the P-gp efflux pump when passing through the cell membrane, thus further increasing the concentration of DTX in A549/ADR cells. TPGS-DTX-liposomes are associated with decreased DTX toxicity, increased safety, reversal of MDR, and improved lung cancer therapy. Wang *et al.*[121] prepared a novel pluronic hybrid paclitaxel-loaded liposomes (PPL). They insert Pluronic P105, a

Table 3. Examples of nanocarriers as drug delivery vehicles for cancer treatment

Category	Characteristic	Composition	Drug	Cell lines	References
Liposomes	Passive targeting	TPGS-liposomes	Docetaxel	A549/ADR cells	[84]
	Passive targeting	Pluronic P105-liposomes-paclitaxel (PPL)	Paclitaxel, ambroxol	A549/ADR cells	[85]
	Active targeting-CD44 receptor	Hyaluronic acid-liposomes	Baicalein, DOX	MCF-7/ADR cells	[114]
	Active targeting-nucleolin	Liposomes-aptamer (AS1411)	DOX	MCF-7/ADR cells	[128]
Polymeric nanoparticles and polymeric micelles	Passive targeting	PLGA	Vincristine, Verapamil	MCF-7/ADR cells	[91]
	Active targeting	ICG-IONP&PLGA-CS&CCM (PIO-PCSCM)	DOX, Mcl-1 siRNA	MCF-7/ADR cells	[130]
	Active targeting-transferrin receptor	PLGA-(D-penicillamine-Au-Cu)-transferrin	-	MDA-MB-231 cells MDA-MB-468 cells	[117]
	Passive targeting	PEG	DOX	MCF-7/ADR cells	[136]
	pH-sensitive	PLGA-TPGS	DOX, Chloroquine	A549/Taxol cells	[149]
Dendrimers	Passive targeting	PAMAM-PEG-DOPE	MDR-1 siRNA, DOX	MCF-7/ADR cells	[98]
	Passive targeting	Pluronic F68 (PF68)-polyamidoamine (PAMAM)	DOX	MCF-7/ADR cells	[100]
	Passive targeting	PAMAM-PEG-DOPE-mAb 2C5	DOX, MDR1 siRNA	MDA-MB-231	[154]
Inorganic nanoparticles	pH-sensitive	Polydopamine (PDA) + MSN + ZIF-8 (PDAMSN@ZIF-8)	DOX, Curcumin	MCF-7/ADR cells	[106]
	Passive targeting	Trimethoxysilylpropyl-ethylenediamine triacetic acid (EDT)-IONPs	DOX	MDCK-MDR1 cells	[107]
		graphene oxide (GO)-molecular beacons (MBs)	DOX	MCF-7/ADR cells	[109]
	pH-sensitive	Au nanorod cluster (AuCR)	DOX, Curcumin	MCF-7/ADR cells	[111]
	Passive targeting Photothermal therapy	Phospholipid-poly(ethylene glycol)-multiwalled carbon nanotubes (Pab-MWCNTs)	P-gp antibodies	3T3-MDR1 cells NCI/ADR-RES cells	[137]
	Active targeting-MUC1	MSN-MUC1	Navitoclax, S63845	MDA-MB-231 cells	[142]
	Active targeting	Cancer cell membrane (CCM)-calcium carbonate (CC)	MiR-451, DOX	BIU-87/ADR cells	[155]
	Active targeting-CD44 receptor photothermal chemotherapy	Molybdenum disulfide-(MoS2)	DOX	MCF-7/ADR cells	[160]
	Passive targeting photodynamic therapy	Acetylated chondroitin sulfate (AC-CS)	Protoporphyrin IX, DOX, Apatinib	MCF-7/ADR cells	[165]
Protein nanoparticles	Active targeting-folate receptor	folate-human serum albumin (FA-HSANPs)	Taxol, 2-ME	EC109/Taxol cells	[115]
	Long internal circulation time	Bovine serum albumin (BSA)	Docetaxel, Quercetin	MDA-MB-231 cells	[147]
	Active targeting-EGFR	Human serum albumin (HSA)	DOX, MDR1 siRNA, Cetuximab	MCF-7/ADR cells	[156]
DNA/RNA	Active targeting-MUC1	MUC1 aptamer-(P-gp and Bcl-2) antisense oligonucleotides	DOX	MCF-7/ADR cells	[127]
Others	Active targeting-folate receptor	Folate-planetary ball milled (PBM) nanoparticles	Resveratrol, Docetaxel	PC3/ADR cells	[175]
	Passive targeting Photodynamic therapy	Lipid-coated carbon-silicon hybrid nanoparticles (LSC)	DOX	NCI/RES ADR cells	[161]

ADR: Adriamycin; CD44: cluster of differentiation-44; DOPE: 1,2-dioleoyl-glycero-3-phosphoethanolamine; DOX: doxorubicin; ICG: indocyanine green; IONP: iron oxide nanoparticle; MCF-7: breast cancer cells; MDCK: madin-darby canine kidney; MDR: multidrug resistance; ME: mercaptoethanol; MSN: mesoporous silica nanoparticle; MUC1: mucin 1; NCI/RES ADR cells: a multidrug-resistant ovarian cancer cell line; PEG: polyethylene glycol; PLGA: poly lactic-co-glycolic acid; PLGA-CS: (poly lactic-co-glycolic acid)-chitosan; RES: reticuloendothelial system; TPGS:

tocopheryl polyethylene glycol succinate; ZIF: zeolitic imidazolate frameworks.

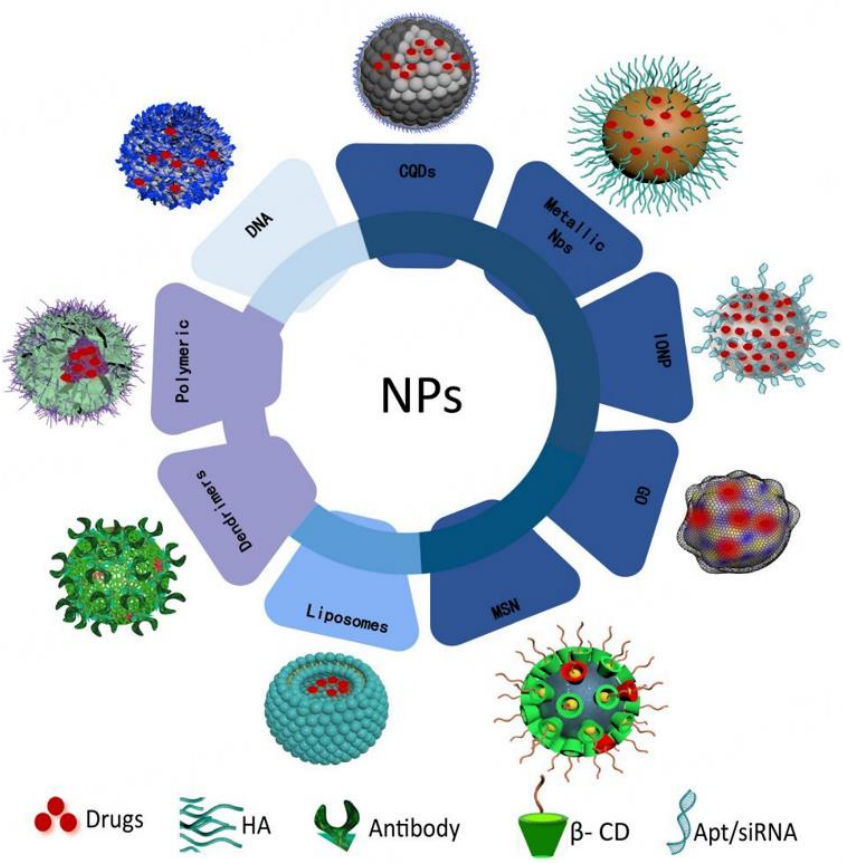

Figure 2. The application of nano-loaded drug system in anti-drug resistance of tumor cells. The advantage of EPR effects could increase the aggregation of drugs in cells, surface-modified antibodies, aptamers, and folic acid can increase the targeting effects, and drugs can also be encased in nanoparticles to escape the recognition of efflux proteins. CQDs: Carbon quantum dots; EPR: enhanced penetration and retention; GO: graphene oxide; HA: hyaluronic acid; IONP: iron oxide nanoparticle; MSN: mesoporous silica nanoparticle; NPs: nanoparticles; β-CD: β-cyclodextrin.

P-gp inhibitor, into the phospholipid bilayer of liposomes and simultaneously load paclitaxel. In drug-resistant A549/Taxol and MCF-7/ADR cells, it can be observed that the PPL + ambroxol regimen has better MDR cell sensitization and killing effect in vitro and in vivo. The Ax can inhibit cell autophagy, while Pluronic P105 can reduce the expression of P-gp. This drug delivery system can increase the retention time of the drug in the lung and resensitize the drug-resistant tumor cells. However, the disadvantage of liposome nano preparation is that its stability is relatively poor, and sometimes the drug leakage occurs after the loading of the drug, resulting in toxicity in vivo[119].

Polymeric nanoparticles and polymeric micelles

Polymer nanoparticles have good biocompatibility, biodegradability, and high drug-loading efficiency[122]. Commonly used polymer materials include artificial synthesis and natural availability. The artificial synthetic materials include polyethyleneimine (PEI), polylysine (PLL), polycaprolactone (PCL), poly lactic-co-glycolic acid (PLGA), and polyamidoamine (PAMAM)[123-126]. Chen *et al.*[127] designed PLGA nanoparticles to deliver vincristine (VCR) and verapamil (VRP). The synergetic effect of chemotherapy drug proliferation is significantly enhanced in MCF-7/ADR cells. The same results are observed in the

MCF-7/ADR tumor-bearing xenotransplantation model in vivo. Coincidentally, people found that micelles have the characteristics of penetrating and accumulating in tumor tissue; therefore, amphiphilic block copolymers have received extensive attention in the development of micellar delivery systems[128,129]. In Guo's[130] work, he developed an intelligent drug delivery system (DDS) to treat drug-resistant BC. The photothermal and magnetic effects of indocyanine green (ICG)-iron oxide nanoparticles (PIO NPs), DOX, and myeloid cell leukemia-1 siRNA (Mcl-1 siRNA) were encapsulated in PLGA-CS NPs. The above NPs were coated by the cancer cell membrane (CCM) of MCF-7 cells to prepare PIO-DOX-siRNA-PCSCM NPs. CCM-encapsulated intelligent drug delivery system (DDS) showed exceptional targeting. At the same time, pH stimulation and near-infrared irradiation accelerate drug release. The efflux of Dox was reduced by NP-treated MCF-7/ADR cells under a magnetic field and near-infrared laser irradiation. And the in vitro cytotoxicity was increased. Drug encapsulation in micelles can improve drug solubility, prolong blood circulation time, reduce side effects, and enhance antitumor activity. So far, many micelles have entered clinical trials or obtained clinical approval. For example, Poloxam, a Planck block copolymer, has been widely used to deliver small-molecule hydrophobic drugs, has been widely used to deliver small-molecule hydrophobic drugs. Genexol-PM polymer micellar nano preparations have been applied for the market and the FDA has also approved the clinical trial[131,132].

Dendrimers

Dendritic polymer is a type of polymer nanocarrier with a star or branch structure that allows therapeutic or diagnostic agents to couple on its surface to maximize the role of tumor diagnosis and treatment[133,134]. Pan *et al.*[135] coupled the fourth-generation dendrimer amino polyamide amine (G4 PAMAM) with a polyethylene glycol (PEG) modified 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PEG-DOPE) block copolymer. Amphiphilic block copolymers can be self-assembled into nano micelles, which can be co-loaded with siRNA onto PAMAM groups and encapsulate water-soluble chemotherapy drugs in hydrophobic cores. This dendrimer micelle mixing system co-delivers MDR-1 siRNA and drugs to tumor tissues through the EPR effect. The positive charge of PAMAM can promote siRNA complexation and tumor cell uptake. The DOPE residues and tertiary amines in PAMAM can improve the escape of endosomes and the intracellular transport of active components[136]. Wang *et al.*[137] designed a series of novel amphiphilic dendrimer micelle (PAMAM-n-PF68), and the core of the new dendrimer has large voids and a higher drug loading efficiency. The drug-loaded NPs can effectively inhibit drug-resistant cells by increasing drug uptake and reducing drug efflux. In vivo studies show that the dendrimer can not only increase the survival rate of nude mice bearing MCF-7/ADR tumors but also reduce the cardiotoxicity of DOX.

Inorganic nanoparticles

Nanocarriers of inorganic materials have been widely developed and studied by scholars due to their unique physical and chemical properties (such as optical, electrical, and magnetic properties, etc.), diversity of structure and function, outstanding biocompatibility and excellent biological distribution, and their ability to overcome bio-barriers at the cellular and tissue levels[138,139]. Among inorganic nanoparticles, magnetic nanoparticles, mesoporous silica nanoparticles, carbon nanoparticles, quantum dots and nano-gold have been widely recognized and concerned by scholars as antitumor drug carriers due to their respective characteristics and advantages[140-142]. Wang *et al.*[143] prepared a core-shell nanocomposite, polydopamine (PDA) mediated integration of the mesoporous MSN core and the microporous zeolite imidazolate frameworks-8 (ZIF-8) shell (PDAMSN@ZIF-8), ZIF-8 and curcumin (CUR, a P-gp inhibitor) were used for plugging to prevent the premature release of DOX from the mesoporous core. Under acidic conditions, the drug-loaded nanoparticles can release CUR and DOX successively, and the CUR is preferentially released into the cytoplasm to inhibit the efflux of P-gp. These NPs realize the high capacity load of DOX and with continuous drug release characteristics.

Iron oxide nanoparticles (IONPs) are one of the most common inorganic nanocarriers. They are biocompatible and can be incorporated into the iron cycle after being degraded in vivo. Norouzi *et al.*[144] developed nanoparticles with biocompatible IONPs stabilized with nanoparticles of trimethoxysilylpropyl-ethylenediamine triacetic acid (EDT). The drug delivery system can provide magnetic targeting for specific sites. At the same time, it prolongs the circulation time of DOX in vivo and increases DOX aggregation in MDR glioma cells.

Among the numerous carbon-based nanoparticles, graphene oxide (GO) has attracted much attention due to its favorable biocompatibility and tolerable toxicity as a nanocarrier for drug delivery[145]. In Li's research[146], a new drug-loaded nanosystem consisting of graphene oxide (GO) modified with two molecular beacons (MBs) and DOX was developed. When the nanosystem was uptaken, DOX was released into acidic endosomes, and MBs modified on the surface of GO can silence intracellular multidrug resistance 1 (MDR1) mRNA and upstream erythroblastosis virus E26 oncogene homolog 1 (ETS1) mRNA, effectively inhibit the expression of P-gp, further prevent DOX efflux.

Gold nanoparticles (AuNPs) are ideal nanocarriers with a small volume ratio, large surface area, high biosafety, easy synthesis, and strong reactivity to cells. Even more remarkable, the gold nanoparticles showed photothermal effects when irradiated by a near-infrared laser at 808 nm. Compared with a single gold nanoparticle, the photothermal or photoacoustic imaging effect of its clusters or combinations is more prominent, with improved drug loading ability, enhanced tumor targeting, and increased drug accumulation in tumors[147]. Wang[148] prepared an Au nanorod Cluster (AuCR) using the emulsion/solvent evaporation method by self-assembly of DOX and amphibious poly (curcumin-co-dithiodipropionic acid) b-biotinylated poly(ethylene glycol) with Au nanorod (AuNR). The AuCR can be decomposed into an independent AuNR unit at an intracellular concentration of 5 mM GSH to trigger DOX release at pH (pH 6.8 or 5.0). The system can increase DOX uptake in ADR tumor cells and inhibit DOX exportation.

NPS TARGETING THE DRUG-RESISTANT TUMOR CELL BIOMARKERS-ACTIVE TARGETING

Active targeting is a high-performance method to deliver the "cargo" (such as therapeutic drugs and genes) into drug-resistant tumor cells by specific targets without affecting healthy tissues, with better therapeutic effects and less toxicity[149]. The outermost layer of drug-loaded nanoplatforms with active targeting functions mainly consists of different types of targeted ligands, such as antibodies, peptides, aptamers, and small molecules. Generally, folate, hyaluronic acid, cell-penetrating peptide, transferrin, and biotin are common targeting modification molecules[150]. For instance, Liu *et al.*[151] prepared hyaluronic acid-modified liposomes containing baicalein and DOX. In vivo and in vitro studies show that compared with DOX alone, the prepared target functional nanoparticles have a stronger inhibitory effect on the proliferation of breast cancer-resistant MCF-7/ADR cells. Liu *et al.*[152] prepared folate-bound human serum albumin nanoparticles (FA-HSANPs) that carry taxol and the chemical sensitizer 2-methoxyestradiol (2-ME). Due to the combination of folic acid and folic acid receptors, the drug-loaded nanoparticles entering EC109/Taxol cells are increasing. The co-encapsulation of PTX and 2-ME in FA-HSANPs can prolong the circulation time, improve the antitumor effect, and reduce the side effects of PTX. It also exhibits highly potent cytotoxicity and apoptosis-inducing activities in the G2/M phase of PTX-resistant EC109/Taxol cells. Meanwhile, 2-ME can effectively reduce the expression of drug resistance-related proteins in EC109/Taxol cells and reverse its MDR to PTX to some extent.

Epithelial-mesenchymal transformation (EMT) in solid tumors promotes tumor progression, MDR, migration, and differentiation[153]. Shome *et al.*[154] used Poly (D,L-lactic-co-glycolic acid) (PLGA) as the

core, the secondary layer covered with Au-Cu nanoclusters, and the outermost layer are transferrin. It can target the transferrin receptor overexpressing triple-negative breast cancer (TNBC) and have available cell internalization ability as well as highly anti-cell proliferation ability. In addition, in EMT-induced TNBC cells, reactive oxygen species (ROS) are produced after co-incubation with nanocomposites, which can induce apoptosis by altering mitochondrial membrane potential. At the same time, the down-regulation of EMT reduced the migration ability of TNBC cells. A 4.63-fold reduction in ABCC1 expression was observed in MDA-MB-231 cells.

The aptamer is a kind of short-chain oligonucleotide with high sensitivity, biocompatibility, biodegradability, and poor immunogenicity. By modifying aptamer sequences on the nanoparticles, selective active targeting of ligands can be achieved[155,156]. Moreover, aptamers have the advantages of convenient synthesis, chemical modification, and high stability. In this regard, some tumor biomarkers that can be detected by targeting ligands include epithelial cell adhesion molecule (EpCAM), mucin-1 (MUC1), nucleolin, prostate-specific membrane antigen (PSMA), HER2, etc.[157-160]. Recently, many targeted aptamers have been studied, including AS1411, 5TR1, HApt, and so on[161-163]. Pan *et al.*[164] used DNA origami technology to assemble MUC1 aptamer functionalized rectangular origami (Apt-origami) with P-gp and Bcl-2 antisense oligonucleotides (Apt-origami-ASO) and then load Dox (Apt-DOA). This Apt-DOA shows good lysosomal escape and drug-responsive release in Hela/ADR and MCF-7/ADR cells. Simultaneously, it can also significantly silence Bcl-2 and P-gp proteins and induce cell apoptosis.

Nucleolin is a membrane shuttle protein that is overexpressed in the cytoplasm and cell membrane in metastatic and rapidly dividing breast cells. Li *et al.*[165] developed a nucleolin-targeting delivery system [Lip (Ap-Dox)] with AS1411-DOX on the inner layer and liposome on the outer layer to avoid drug resistance in breast cancer (MCF-7/Adr). The advantage of the above NPs is the transmembrane affinity of liposomes to deliver nanoparticles into cells and release Ap-Dox, which increases the accumulation of Ap-Dox in cancer cells. In addition, this targeting of AS1411-nucleolin can accumulate Dox in the nucleus, thus highly enhancing its therapeutic efficacy against drug-resistant cancer cells by effectively bypassing P-gp. This novel nano-drug treatment strategy for breast cancer provides different insights into the future design of NP-delivery drugs.

NPs target efflux and transfer system to overcome tumor resistance

One way to overcome the MDR controlled by ABC transporter is to use ABC transporter inhibitors to make tumor cells sensitive to chemotherapy drugs. ABC transporter inhibitors were combined with anticancer drugs to improve drug sensitivity. However, the toxicity of the MDR reversal agent limits its application. Nevertheless, the enthusiasm for ABC efflux transporters to overcome MDR is still high. Delivery of drugs into cells by employing a nanocarrier can enhance the accumulation of nano drugs in cells and reverse the efflux of ABC transporters. This method has become one of the most effective methods to overcome the drug resistance of tumor cells[166].

Currently, commonly used MDR effector inhibitors include cyclosporine, verapamil, Taliqueta, and curcumin[167,168]. The scientists also found that some surfactants also have anti-MDR effects, such as Solutol®HS15, Cremophor®EL, Tween®80, and TPGS, all of which contains PEG in their hydrophilic parts.[167,169-171]. These nanoparticles are also good carriers for drug delivery. Zhang *et al.*[172] studied the effect of the surface charge of PAMAM dendrimer on the exocytosis and mechanism of multidrug-resistant tumor cells. In drug-resistant human breast cancer cells (MCF-7/ADR cells), exocytosis kinetics, pathway, and mechanism were systematically analyzed. The results prove that the positive charge on the surface of PAMAM dendrimers promoted exocytosis, while the neutral and negatively charged dendrimers had a

slight effect on exocytosis in MCF-7/ADR cells. PAMAM-NH2 with positive charges is more likely to be distributed in mitochondria and nuclei, which is an ideal mitochondrial targeting agent and gene-drug carrier for multidrug-resistant tumor therapy.

Mao *et al.*[173] designed polyethylene glycol (PEG, MW: 2K) to deliver DOX (PEG_{2K}-DOX). PEG_{2K}-DOX nanoparticles significantly increased the uptake and cytotoxic activity of MCF-7/ADR and KBv200 to DOX, and their IC50 values decreased to 1.130% and 42.467%, respectively. Compared with doxorubicin, PEG_{2K}-DOX nanoparticles shows significantly improved plasma pharmacokinetics, enhanced in vivo therapeutic efficacy against MDR xenografts, and better safety in vivo. Suo *et al.*[174] has studied and synthesized a multiwalled carbon nanotubes dense coating with phospholipid-poly(ethylene glycol) (Pab-MWCNTs). By connecting MWCNTs to P-gp antibodies, the combination of body targeting and laser-guided heat therapy was realized. Pab-MWCNTs can penetrate deeply within tumor spheroids, but after brief exposure to near-infrared, both P-gp-transfected 3T3 cells and P-gp-expressing drug-resistant cancer cells showed higher lethal activity. Singh *et al.*[175] prepared a novel nano-drug system called the planetary ball milled (PBM) nanoparticles (FA-RES + DTX-NP) that were coated with resveratrol (RES), combined with docetaxel (DTX), and surfacing coupled with folic acid (FA), which could enhance the bioavailability of resveratrol (RES) and DTX to treat advanced metastatic PCa. Planetary milling technology has attracted more and more attention for the advantages of preparing nanoparticles without additive or final dewatering steps to recover nanoparticles. Over the years, the ball milling process has been explored for nano reduction of different polymer materials, including starch, cellulose, and protein-based polymers, for different applications[176,177]. The cytotoxic effect of FA-RES + DTX-NP nanoparticles is very effective and can reduce the concentration of free drug (DTX) by 28 times. After FA-RES+DTX-NP treatment, the expressions of NF-kB p65, COX-2, pro (BAX, BAK), and anti-apoptotic (Bcl-2, Bcl-XL) genes were significantly decreased. In addition, treatment of DTX-resistant PCa cells with FA-RES + DTX-NP negatively affected the ABC transporter marker, thereby limiting the multidrug-resistant phenotype of cancer cells and effectively enhancing the intracellular concentration of the drug, thereby exerting its cytotoxic effects.

NANO DRUG DELIVERY STRATEGY FOR REGULATING APOPTOSIS AND METABOLISM-RELATED FACTORS

In the human body, apoptosis is a highly programmed cell death process[178]. When apoptosis is defective, it may also lead to drug resistance to chemotherapy drugs. And this defect may be caused by the inactivation of apoptosis-promoting genes and activation of anti-apoptotic proteins of the Bcl-2 protein family, as well as survival signal proteins (Survivin, FLIP, and NF- κB)[65]. In the development of fighting against drug resistance of tumor cells, people have studied the proteins related to the regulation of apoptosis and developed inhibitors and drug-loaded nanoparticles that can interfere with the process of apoptosis. Recently, inhibitors of Bcl-2 and MCL-1 anti-apoptotic proteins have been developed to overcome drug resistance, such as ABT-263 (Navitoclax) against Bcl-2 and S63845 against MCL-1[179-181]. For example, Vivo-Llorca *et al.*[182] prepared MSN-apMUC1 containing Navitoclax and S63845. By modifying the transmembrane protein MUC1 aptamer overexpressed in breast cancer cells on the surface of mesoporous silica nanoparticle (MSN), the system can be actively targeted to the MDR cells. In addition, the MSN gap also carries an inhibitor of anti-apoptotic proteins. The drug-loaded nano platform could effectively target Triple-Negative Breast Cancer (TNBC) cells and successfully induce apoptosis. The drug delivery system improves the antitumor activity and is effectively targeted to TNBC cells.

The down-regulation of the *C-Myc* gene contributes to the occurrence of a series of human tumors by regulating endogenous and exogenous apoptosis pathways[183]. In Wang's[184] study, it was confirmed that multiwalled carbon nanotubes (MWCNTs) could reduce the expression of ABC transporter in human

colorectal adenocarcinoma Caco-2 cells by regulating the expression of C-Myc. Overexpression of C-Myc reversed the inhibitory effect of MWCNT on ABCB1 and ABCC4 expression.

COMBINATION-BASED NANOPARTICLE APPROACHES DIRECTED AGAINST MDR

Nano-drug platform co-delivers chemotherapeutic drugs and sensitizers

In addition to the emerging P-gp inhibitory nanomaterials mentioned earlier, some products extracted from natural plants have also been gradually proven to have anti-P-gp efflux effects. These natural products mainly include alkaloids, coumarins, flavonoids, and terpenes[185]. For example, quercetin (QT) in flavonoids has been shown to induce apoptosis, inhibit angiogenesis, and inhibit oxidative stress and mutation in several human cancer cells[186]. Desale *et al.*[187] synthesized a novel intravenous bovine serum albumin (BSA) co-loaded Docetaxel (DTX) and quercetin (QT) nanoparticle (DTX-QT-BSA-NPs). In this drug delivery platform, BSA plays a role in prolonging the circulation time of nanoparticles in vivo and the active targeting of NPs to tumors. QT is used as a chemical sensitizer and is first released to inhibit the action of P-gp efflux transporters. The in vivo assay demonstrates that the two drugs significantly enhance the accumulation of DTX in MDA MB-231 (P-gp efflux sensitive) cells by nanoparticle encapsulation compared with free DTX and DTX-BSA-NPs. DTX-QT-BSA-NPs increased the titer of therapeutic drugs in tumor cells.

In recent years, scientists have observed that the anti-malarial drug, chloroquine (CQ), is an inhibitor of lysosomes. CQ can inhibit the fusion of lysosomes and affect the degradation of late autophagy[188]. It is a potential natural chemosensitizer. Sun *et al.*[189] prepared Poly(lactic-co-glycolic acid) (PLGA) and D-alpha-tocopheryl polyethylene glycol succinate (TPGS) as carriers to co-delivered DOX and CQ. As a chemical sensitizer, CQ can increase the accumulation of drugs in cancer cells by protecting DOX from autophagy degradation. NP_{DOX+CQ} is pH-sensitive and stable in the neutral environment of normal tissue cells and blood but dissociates in the weakly acidic environment of tumor cells and releases loaded drugs, thus achieving antitumor effects. More importantly, NP_{DOX+CQ} can avoid the recognition of P-gp and thus reduce drug resistance.

Nano platform co-delivers chemotherapy drugs and genes

Co-delivery of antitumor drugs and genes can improve the effectiveness of treatment. On the one hand, genes can increase the drug sensitivity of tumor cells and thus can reduce the drug's adverse reactions. On the other hand, it can overcome the problem of low transfection efficiency in gene therapy, which leads to poor therapeutic effects[190,191]. Gene therapy usually refers to the therapeutic genes in human target cells in a certain way to correct or replace disease-causing genes and correct gene defects, play the role of biomedical therapy, and achieve the purpose of treating diseases. Small interfering RNA, small RNA, long non-coding RNA, and small hairpin RNA have shown great potential in current-period gene therapy[192,193]. They play important roles in regulating tumor of multidrug resistance, the expression of cell cycle-related genes, the expression of DNA repair ability-related genes after cell damage, inducing autophagy, and increasing the sensitivity of chemotherapy drugs to targets. Yalamarty[194] prepared a 2C5-modified MDM with siRNA and DOX (2C5-MDM-D-R). Specifically, the tumor-targeting monoclonal antibody 2C5 (mAb 2C5)-PEG_{7k}-DOPE was inserted into a mixed dendritic molecule micelle containing 4th generation (G4) polyamidoamine (PAMAM)-PEG_{2k}-DOPE and PEG_{5k}-DOPE. The MDR1 siRNA binds electrostatically to the cationic charge on the G4 PAMAM dendrite molecule. The micelle has shown active targeting against tumors in breast (MDA-MB-231) and ovarian (SKOV-3TR) MDR cell lines. MDR1 siRNA inhibited the drug efflux and increased the accumulation of drugs in the tumor cells to reduce the off-target effect. In Wei's[195] study, a cancer cell membrane (CM) coated with calcium carbonate (CC) nanoparticles was designed to co-deliver miR-451 and DOX to solve the dilemma in bladder cancer treatment (MCC/R-A). The system can target retention and bypass extracellular barriers. In addition, miR-451 overcomes the MDR

of cancer cells by down-regulating the expression of P-gp, thereby increasing cellular drug retention in BIU-87/Adr. The therapeutic effect of MCC/R-A on BIU-87/Adr was significantly enhanced, which was better than miR-451 or DOX alone. Yang *et al.*[196] used Human serum albumin (HSA) as the carrier to deliver Dox and MDR1 siRNA simultaneously, and the outer layer was modified with cetuximab. Cetuximab in this nano-loaded system targets overexpressed epidermal growth factor receptors (EGFR) in MCF-7 /ADR cells, so DOX and siMDR1 to tumor sites, enhancing gene silencing and cytotoxic activity.

The synergistic effect of immunotherapy and chemotherapy on different mechanisms is a hot research topic in cancer medicine and has a broader clinical application prospect. Immunotherapy by RNA interference is beneficial to long-term block the interaction of PD-1/PD-L1 (Programmed cell death protein 1 and its ligand) and reduce the proliferation activity of cells[197,198]. Tang[199] used mixed micelles to co-deliver Paclitaxel (PTX) and PD-L1 siRNA for melanoma treatment. The mixed micelles could reduce the expression of PD-L1 and p-S6K(mTOR pathway activation markers) in B16F10 cells. Furthermore, cytotoxic T cell immune response is promoted, and mTOR(Mammalian target of rapamycin) pathway activation is inhibited, which can cooperate with chemotherapy to reduce tumor proliferation activity.

Chemotherapeutic drugs combined with photothermal therapy

Near-infrared laser has the advantages of high efficiency in penetrating tissue and safety, which can be used in tumor treatment. Dong *et al.*[200] designed a molybdenum disulfide (MoS_2) modified NPs with hyaluronic acid (HA) by taking advantage of its fine particle size and high photothermal conversion efficiency. Under the irradiation of an 808 nm laser, the temperature of MoS_2 can rise from room temperature to 52 °C within 10 min, which can effectively inhibit the proliferation of drug-resistant tumor cells and significantly reduce the expression of membrane transporters. In vivo experimental observation shows that the tumor growth inhibition rate in the MoS_2-HA + laser group is as high as 96%, indicating that MoS_2-HA/DOX can primely deliver drugs and maintain the in vivo drug concentration, improving the effect of combined photothermal chemotherapy. Wang *et al.*[201] successfully constructed a nano drug delivery system with lipid-coated carbon-silicon hybrid nanoparticles (LSC) as the carrier to carry the chemotherapy drug DOX. Under 780 nm laser irradiation, the nanosystem can target mitochondria and produce reactive oxygen (ROS). The ROS can oxidize NADH into NAD^+ to reduce the amount of ATP available to the efflux pump and promotes MDR cancer cells to return to "normal" for at least 5 days. In vivo data shows that drug-loaded LSC nanoparticles combined with NIR laser therapy can effectively inhibit the growth of multidrug-resistant tumors without significant systemic toxicity. The role of the nanoparticles is also verified in two drug-resistant cell models, paclitaxel-resistant and irinotecan resistant, indicating that this design can effectively overcome the broad-spectrum drug resistance of tumors.

Chemotherapeutic combined with photodynamic therapy

Photodynamic therapy (photodynamic therapy, PDT) is one of the important adjuvant treatments for cancer. Compared with traditional treatment, PDT is less invasive and less damaging to normal tissues. In addition, it can effectively induce immunogenic cell death (ICD) and stimulate immunity, which is an ideal minimally invasive treatment for tumors[202,203]. Photosensitizers mainly include the porphyrin family (hematoporphyrin derivatives, benzoporphyrin derivatives, 5-aminolevulinic acid, Texaphyrins), the chlorine family (Purlytin, Temoporfin, Photochlor), and the dye family (Phthalocyanine, phthalocyanine)[204]. The photosensitizers have limitations in clinical applications due to the following properties: poor solubility in water, low chemical purity, and poor tissue penetration. The combination of photosensitizers and nanomaterials can increase the efficiency of PDT as well as eliminate its side effects. In addition, nanoparticles can simultaneously carry chemotherapy drugs and photosensitizers into drug-resistant tumor cells to synergistically inhibit the proliferation of drug-resistant tumor cells. Wei *et al.*[205] used acetylated Chondroitin sulfate (AC-CS) as a long-cycling

nano craft and combined protoporphyrin IX (PpIX) onto AC-CS (ACP) via an ester bond. DOX and Apatinib (Apa) can be simultaneously encapsulated into amphiphilic ACP micelles (ACP Dox + Apa). When exposed to 635 nm light, PpIX was activated to produce ROS, then the release of DOX and Apa were triggered. At the same time, excessive ROS have a strong PDT effect on mitochondria or nuclei and eventually lead to apoptosis. This novel nanosystem reverses tumor MDR through Apa-enhanced DOX sensitivity combined with photosensitizer-mediated PDT.

CONCLUSION

Through continuous efforts, scientists have made great progress in uncovering the mechanisms of drug resistance in cancer. The occurrence of MDR in tumor cells is mainly due to overexpression of transmembrane proteins, abnormal signal transduction pathways, mutations in drug targets or related enzyme systems, dynamic activation of the DNA repair system, and the developing adaptation of cancer cells to the microenvironment, etc., which leads to reduced drug sensitivity. The mechanism of MDR in tumors is very complex, and it is difficult to reverse the drug resistance with a single preparation. However, how to overcome cancer resistance is still an unsolved problem. As more nanomedicine is developed and optimized, the advantages of nanomedicine will become an attractive strategy for reversing or overcoming cancer resistance.

The rational design of a nano drug delivery system can enhance drug solubility, system stability, targeting, and biocompatibility. In general, through the improvement and modification of nanomaterials, the drugs it carries can be more internalized into cells, increasing the concentration of drugs in drug-resistant tumor cells. At the same time, different drugs can also combine with proteins on the cell membrane to reduce drug efflux. Furthermore, these drug-loaded nanoparticles can interfere with the metabolism and apoptosis of drug-resistant tumor cells, silence drug-resistant genes, and downregulate the synthesis of drug-resistant related proteins. In addition, nano drug delivery systems can realize the therapy multi-target and multi-pathway combined, which is one of the future development directions of tumor therapy. It indicates that nanomaterials show a highly competitive application prospect in drug delivery of drug-resistant tumor cells. In this paper, we systematically summarize different strategies based on nanomedicine to improve the efficacy of chemotherapy in drug-resistant tumor cells, including enhancing the drug enrichment of chemotherapy drugs, improving pharmacokinetics, controllable modifications, and reversing the drug resistance of tumor cells in combination with chemotherapy. Compared with traditional preparations, nano drugs can maintain stability in systemic circulation after injection, protect drugs from damage utilizing plasma protein binding, increase circulation time, and realize efficient accumulation and function of therapeutic components in tumor sites. Therefore, the nanocarrier drug delivery with unique functionality and systemic circulation stability is a novel strategy for overcoming MDR. Among the drug-loaded nanoparticles mentioned in this paper, compared with traditional drug-delivery system carriers, polymer micelles have a higher drug-loading capacity, longer drug circulation time, and lower systemic toxicity. Liposomes are used as delivery carriers for water-soluble drugs(such as proteins or DNA), while insoluble drugs are surrounded by a hydrophobic bilayer. However, due to the instability of the membrane, drug loading is limited. Fortunately, PEG-modified phospholipids prevent the recognition and uptake of liposomes by RES, prolong the retention time of drugs in the blood circulation, and can be enriched in tumor cells in tissues or organs other than the liver and spleen by the EPR effect[206]. The safe dose of inorganic nanomaterials is uncertain and can cause oxidative stress in normal tissues, which may affect the function of some vital organs. Secondly, most inorganic nanomaterials enter cells through endocytosis, and their uptake by tumor cells directly affects the therapeutic effect. How to improve the targeting of inorganic nanomaterials to tumor tissue is the focus and difficulty of research. In short, the application of nanomaterials in MDR tumors is still in its infancy. The biological safety of nano drug delivery systems still

needs to be further explored. These issues include optimizing nano delivery systems to make them more suitable for human use, avoiding or reducing possible toxic and side effects, and degradation and excretion of nanoparticles. In addition, the methods of reversing tumor drug resistance by the nano-drug delivery system still need to be innovated. The efficiency of nano drug delivery systems in MDR tumors can be improved by combining immunology, photodynamics, acoustic dynamics, and other methods may be a key research strategy for future research.

DECLARATIONS

Authors' contributions

Made substantial contributions to the conception and design of the study: Sun X, Chen K, Shen J
Provided administrative, technical, and material support: Zhao P, Lin J

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Mechanisms and clinical implications in renal carcinoma resistance: narrative review of immune checkpoint inhibitors

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Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of renal cell carcinoma. The prognosis for patients with ccRCC has improved over recent years with the use of combination therapies with an anti-programmed death-1 (PD-1) backbone. This has enhanced the quality of life and life expectancy of patients with this disease. Unfortunately, not all patients benefit; eventually, most patients will develop resistance to therapy and progress. Recent molecular, biochemical, and immunological research has extensively researched anti-angiogenic and immune-based treatment resistance mechanisms. This analysis offers an overview of the principles underpinning the resistance pathways related to immune checkpoint inhibitors (ICIs). Additionally, novel approaches to overcome resistance that may be considered for the trial context are discussed.

Keywords: Renal cell carcinoma, immunotherapy, treatment resistance, tumor microenvironment, intrinsic factors

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 3%-5% of all malignancies in adults, with an incidence of about

400,000 cases yearly and mortality in approximately 175,000 cases worldwide in 2020[1-4]. Around one-fourth of patients present initially with metastasis, with the rest recurring after nephrectomy[5].

The most common histological pattern of renal tumors is clear cell renal cell carcinoma (ccRCC), which accounts for 70%-90% of the disease, followed by papillary (10%-15%) and chromophobe RCCs (3%-5%)[6,7].

In the past few decades, the mainstay of treatment of metastatic RCC (mRCC) involved vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR-TKI)[8,9]. Monoclonal approaches to the same target, as characterized by bevacizumab, have been unsuccessful. A study by Motzer *et al.* showed no improvement in OS in patients with mRCC treated with atezolizumab plus bevacizumab[10]. When this immunologically "hot" tumor microenvironment is exposed to immune checkpoint inhibitors and/or inhibitors of VEGF as a combination, outcomes are significantly and clinically improved compared to VEGFR-TKI monotherapy alone[7,11]. Unfortunately, a cure is still not achievable for the vast majority of these patients presenting in the metastatic setting.

This review aims to summarize the molecular mechanisms that drive resistance to immune checkpoint inhibitors and their clinical implications in patients with mRCC.

Molecular mechanism of resistance to immune checkpoint inhibitors

Resistance to ICIs therapy has been classified into primary (initial) resistance and secondary (acquired) resistance[12]. Part of the classification is based on imaging response to ICI therapy, defined by the Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 criteria[13]. Primary resistance is defined as the best response of progressive disease (PD) or stable disease (SD) for less than 6 months of ICI initiation. Patients with secondary resistance develop progressive disease after having a response or stable disease for greater than six months. Various mechanisms have been postulated for developing primary and secondary resistance in solid tumors, including mRCC, which can be broadly subcategorized into patient-related factors, tumor cell-related factors, or factors related to the tumor microenvironment (TME). The precise role of these mechanisms and a variety of others in primary and secondary resistance in the context of mRCC remains to be fully elucidated. However, a better understanding of these complex mechanisms is crucial to optimizing treatment approaches for those resistant to mRCC disease[14].

Patients'-intrinsic factors

Patients'-intrinsic factors play a vital role in the immune response to the therapy. Several factors are related to the immune response in cancer therapy and are summarized in Table 1.

Gender

Gender-related factors may be attributed to confounding behaviors, such as male patients with increased exposure to smoking[15]. Another possible hypothesis is the role of estrogen modulation. Estrogen upregulates the activities of T-cells and PD-1 expression on cells[16,17]. A recent meta-analysis showed a significant disparity in overall survival (OS) between men and women with metastatic cancers with pooled OS in men was HR = 0.72 (95%CI: 0.65-0.79) and in women was HR = 0.86 (95%CI: 0.79-0.93), with a positive interaction value[15].

In a randomized trial in patients with ccRCC, treated with nivolumab or everolimus in a second-line setting, the hazard ratio (HR) for death in males was 0.70 (95%CI 0.50-0.90) compared to 0.84 (0.57-1.24) in women, which was similar to patients with melanoma or small cell lung carcinoma treated with ICIs[18].

Table 1. Molecular mechanism of resistance - patient's intrinsic factors

Factor	Theorized mechanism	Potential outcomes
Gender[15-18]	Exposure to mutagenic factors (i.e., Smoking, exposure is higher in men)	Differences in tumor histology
	Estrogen modulation leads to: (a) Increased expression of FoxP3 (b) Modulation of PD-1 expression	Increased regulatory T-cell, dendritic cell, and macrophage activity.
Obesity[19]	Both pro-tumor and anti-tumor activity	Mixed outcomes in patients with RCC
Sarcopenia[95]	Low skeletal mass index	Worse prognosticator
HLA genotype[25,26]	Classical HLA-1 Molecules resulting in High Divergent Expression	Greater response to ICI treatment
Gut microbiome[27,28]	Fecal Microbiota Transplant resulting in upregulation of CD4+ T cell and PD-L1	Improved ICI response
Antibiotic/Steroid use[29,33]	Antibiotic Use causing dysbiosis	Decreased ICI response
	Steroids altering T-cell activation, altering gut microbiota	May result in favorable disease course due to other factors

PD-1: Programmed cell death protein 1; HLA: human leucocyte antigen; ICI: immune checkpoint inhibitors.

Obesity

Obesity has shown variable modification capabilities in a human cell with both pro-tumor (increased VEGF, vascular endothelial growth factor, hypoxia, angiogenesis, plasmacytoid dendritic cells, and mast cell infiltration) and anti-tumor activities (increased T-cell and natural-killer cell response and decrease intratumor PDL-1 expression)[19]. Several studies have shown the beneficial effects of obesity on survival in patients with RCC[20,21]. Utilizing CT based body composition, correlations with specific phenotypes were found (i.e., Adipose, skeletal density vs. sarcopenia). While the exact mechanism to an increased BMI results in positive outcomes, theories include tumorigenic immune dysfunction *vs.* T-cell antigen cross-reactivity. This has the potential to improve sensitivity to ICI therapy. In contrast, an analysis of those with a similar phenotypic profile who were receiving Anti-PD-1 therapy showed a reduction in therapeutic response (lower PFS) as compared to their leaner counterparts[22-24].

Malnutrition and sarcopenia

Sarcopenia has been associated with adverse outcomes even before the immunotherapy era[22-24]. A retrospective multicenter real-world study found that sarcopenic patients had significantly worse OS (HR = 2.2, 95%CI: 1.3-3.6, P = 0.0026) when treated with ICIs. On multivariate analysis, low muscle mass was associated with an inferior OS[25]. Similarly, malnutrition has shown poor prognosis and quality of life in patients with lung cancer and is useful in predicting the response to the treatment[26,27].

Human leucocyte antigen genotype

Human leucocyte antigen (HLA) class 1 genes usually underlie the control of cancer with the diverged presentation of proteins that can influence response to ICIs[25,26]. This hypothesis suggests that patients with heterozygous divergent alleles have a more extensive presentation of peptides to assist T-cell response than less divergent HLA alleles. Patients with melanoma having high divergent alleles receiving ICIs respond better to the treatment. The outcomes were significantly different with 20 months in patients with high divergent alleles than 8 months in patients with low-divergent alleles (HR = 0.43, 95%CI: 0.2-0.8, P = 0.0094)[26]. Similar outcomes were observed in non-small cell lung cancer patients who received ICIs[25,26]. So far, there is a lack of specific studies for patients with RCC to understand the difference between high versus low divergent alleles and their response to the treatment[25,26].

Gut microbiota

Fecal microbiota transplantation (FMT) has been studied as a predictive factor for a response to ICIs treatment. Routy *et al*. found a positive correlation between response to ICI and Akkermansia muciniphila in mice receiving anti-PD-1 blockade[27]. It has also been noticed that the FMT from a patient who responded to ICIs in germ-free mice improves ICI efficiency but, unfortunately, not in non-responding patients. FMT upregulates helper T-cells (CD4+) and natural killer T- cells (NKT) in the spleen, resulting in improved response to ICIs. In a prospective randomized control trial, the gut microbiome modulation in patients with mRCC has been associated with improved PFS[28], which will be discussed later in this paper.

Antibiotics and use of steroids

The alteration in the gut microbiome predicts the response to immunotherapy. It has been observed that dysbiosis with antibiotics has been associated with poor outcomes in patients treated with ICIs[29]. Other retrospective studies by Lalani[30], Derosa *et al.*[31], and Tinsley *et al.*[32] also showed a negative association between antibiotic use and outcomes in patients with RCC, with a significant decrease in PFS[30-32]. Derosa *et al.*[31] and Tinsley *et al.*[32] also showed a reduction in OS. On multivariate analysis, antibiotic use was an independent predictive factor for worse outcomes in patients with RCC[31,32].

The use of steroids is routine in oncology patients for symptom management. A dose of > 10 mg prednisone or equivalent induces immunosuppression by altering the T cell activation with the expansion of M2 macrophages and thus altering the gut microenvironment. In a multicentre study by Arbour *et al.*[33], baseline higher steroid dose was associated with lower ORR (7% *vs* 18%), progression-free survival (PFS) ($P < 0.001$), and OS ($P < 0.001$). However, steroids used to manage immune-related adverse events have shown no negative impact on the efficiency of ICIs[34-36]. Indeed, patients that develop severe immune-related adverse events and need systemic steroids have a more favorable disease course in recent real-world data[37].

TUMOR CELL INTRINSIC FACTORS

Tumor cell-intrinsic factors are cell-related factors that can identify the response to the therapy and are summarized in Table 2.

Signalling pathways

Interferon-gamma signaling pathway

The activation of interferon-gamma (IFN-γ) activates Janus kinase (JAK), and interferon regulatory factor 1 (IRF1), leading to the expression of PDL1. Patients with dysregulation of the IFN-γ pathway were shown to develop resistance to ICIs[38]. The IFN-γ recruits immune cells and enhances MHC-1 antigen presentation, which is essential for the antiproliferative and proapoptotic signals[39,40]. The dysregulation of JAK genes has been associated with a lack of response to the IFN-γ and, thus, with PD1 inhibitors[41].

Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPK) is associated with VEGF, IL-6, IL-8, and IL-10 production, inhibiting T-cell functions and immune cell recruitment. The MAPK pathways downregulate antigen presentation and MHC expression and decrease the sensitivity to antiproliferative effects of IFN-γ and TNF alpha[38,42].

PI3K/mTOR/AKT pathway

Patients with ccRCC have altered PI3K/AKT pathway along with alteration in the expression of PTEN[43]. These lead to the inhibition of immunosuppressive cytokines and auto phagosome, resulting in decreased T cell response, cell recruitment, and cell-mediated death. The loss of PTEN has been associated with the

Table 2. Molecular mechanism of resistance - tumor cell-intrinsic factor

Factor	Theorized mechanism	Potential outcomes
IFN-γ dysregulation[39-41]	Loss of JAK-1&2 Function resulting in decreased PD-L1 expression	Resistance to PD1 Inhibitors
	Decreased immune cell recruitment with decreased MHC-1 antigen presentation	Increased proliferation and decreased apoptosis
MAPK[38,42]	Increased production of VEGF, IL-6,8 and 10 inhibiting T-cell function	Increased resistance
	Downregulation of antigen/MHC expression	Increased proliferation and decreased apoptosis
PI3K/AKT alteration[43,44]	Loss of expression of PTEN resulting in: Cytokine suppression Inhibition of auto-phagosome activity	Decrease ICI response due to poor recruitment.
Wnt/β-catenin overexpression[45,46,48]	Absence of T-cell expression and T-cell exclusion	Resistance to ICI therapy
Loss of MHC[96-99]	Loss of MHC-1 and 2	Resistance to ICI therapy

IFN-γ: Interferon-gamma; PD-L1: programmed death ligand 1; JAK: janus kinase; MHC: major histocompatibility complex; VEGF: vascular endothelial growth factor; IL: interleukin; PI3K: phosphoinositide 3-kinases; PTEN: phosphatase tensin homolog; ICI: immune checkpoint inhibitors; MAPK: mitogen-activated protein kinase.

worse outcome in patients treated with ICIs therapy[44].

Wnt/β-catenin pathway

Wnt/β -catenin is involved with stem cell embryogenesis, immune regulation, and cell differentiation. In most cancers, it is overexpressed, reducing the expression of T cells and thus resulting in resistance to ICIs[45-48].

Insufficient tumor antigenicity

Investigations highlighted a correlation between poor antigenicity and decreased sensitivity to ICIs[49,50]. Repeat analysis supports the idea that these tumor neoantigens can be targeted to promote a positive response to checkpoint blockade. This concept can be applied to a variety of malignancies.

It is important to highlight that despite an understanding of the molecular patterns of resistance to ICIs in this context, no current biomarkers, molecular or otherwise, are validated for use in the clinic to help determine therapy selection.

TUMOR MICROENVIRONMENT

Tumor microenvironment (Tme) includes tumor cells and other cells interacting with them. These cells are crucial for tumor cells' development, progression, and relapse. Factors that can affect the TME are summarized in Table 3.

Tumor-associated macrophage

During inflammation, macrophages transform to M1 (classical) or M2 (alternative) activation. M1 produces inflammatory cytokines (ie. IL-6, IL-12, and IL-23), whereas the later correlates with the subsets of M2a, M2b, M2c, and M2d, respectively[51,52]. In 2011, a study found poor survival in patients with RCC with a high burden of M2 (CD163+)[53]. The diverse and extensive tumor-associated macrophages in the TME of patients with RCC indicate cancer progression and metastases. Strategies to direct therapeutic response to suppress tumor-associated macrophage recruitment have shown some improvement in the response[54], but macrophage-targeted therapies still need to be implemented in clinical settings

Table 3. Molecular mechanism of resistance - tumor microenvironment

Factor	Theorized mechanism	Potential outcomes
Tumor-associated Macrophage activation[51-53]	Greater M1 and M2 Macrophage activation, M2 (CD163+) subset correlated to poor survival outcomes	Nivolumab improved PFS in high M2 density activation
High T-cell density[56]	Higher CD8+ infiltration associated with poorer clinical outcomes in ccRCC patients	Prognosticator for worse potential outcomes
B-cell and tertiary lymphoid structures[50,57,62]	Increased B cell-related genes associated with strong memory response	Great response to therapy
	Tertiary lymphoid structures promote Regulatory T-cell activation	Prognosticator for positive potential outcomes.
Hypoxia[64-67]	Increased recruitment and infiltration of myeloid-driven suppressor cells, decreased function of cytotoxic T-cells	Immune evasion
	Induction of HIF-1a and 2a, increasing PD-L1 expression, and VEGF generation	Immune evasion

PFS: Progression-free survival; ccRCC: clear cell renal cell carcinoma.

.

The TME associated with abundant cells has shown an overall better response to ICIs ($P < 0.001$) as compared to TKI treatment ($P = 0.15$)[55]. Voss *et al.* found that the TME, with abundant infiltrative cell types, was associated with a good response to ICIs ($P < 0.001$), which was the opposite in patients treated with TKIs ($P = 0.15$)[55]. In another trial, patients treated with nivolumab having higher densities of M2 macrophages showed better PFS (HR = 0.69, $P = 0.016$), but no significant improvement in OS ($P = 0.5$)[56].

T cells

RCC is a tumor mainly enriched with T cells, particularly CD8+ T cells. The presence of CD8+ T cells is correlated with poor outcomes[57,58]. Choueiri *et al.* reported that high CD8 infiltration was associated with poor clinical outcomes in patients with ccRCC treated with sunitinib versus the avelumab-axitinib combination[59]. This was discordant from another phase II study, NIVOREN-GETUG AFU 26, which showed that patients with high CD8+ infiltration treated with Nivolumab have poor PFS (HR = 3.96, $P < 0.0001$) and OS (HR = 2.43, $P = 0.04$)[56]. Interestingly, Voss. *et al.* identified no correlation between the CD8+ cell infiltrations and survival in mRCC when treated with ICIs[55].

The Cancer Genome Atlas analysis showed that patients with RCC have more regulatory T-cells and are associated with poor clinical outcomes (HR = 1.59, 95%CI: 1.23-0.06; P < 0.01)[60].

B cells and tertiary lymphoid structures

B cells assist T-cell regulation and differentiation through different pathway activation, including interleukin (IL) and growth factors[49]. The memory of classical B cells has been associated with response against tumor-associated antigens[50]. The Microenvironment Cells Populations-counter (MCP-counter) analysis showed that responders have higher B cell densities in the TME than non-responders in melanoma and ccRCC[61].

Tertiary lymphoid structures are also associated with better outcomes in oncology patients. It assists in transforming cells and activating T regulatory cells[57,62].

Hypoxia

Tumors in the hypervascular environment led to insufficient nutrition and oxygen intake, resulting in hypoxia and progression[63]. Hypoxia induces upregulations of myeloid-driven suppressor cells that lead to

decreased function of cytotoxic T cells[64,65]. Hypoxia also induces factor 1a (HIF-1a) and 2a (HIF-2a) to express PD-L1 in tumor cells[66,67]. Elevated levels of HIF are associated with generating VEGF, which acts as an immune escape mechanism

by upregulating CTLA4, TIM3, LAG3, and PD-L1 on dendritic cells[65,68]. Hypoxia causes tissue deposits of adenosine which suppresses the activity of T cells[69].

CLINICAL IMPLICATION OF ICIS RESISTANCE

ICI resistance in the clinical trial setting

Despite therapeutic advances, ICI resistance remains a significant issue in clinical trials and real-world settings due to a lack of validated clinical tools to identify patients at risk for primary or secondary resistance. Currently, there is no clinically accessible molecular classification of mRCC, and although clinical trials investigating molecular biomarkers in mRCC populations are promising, such approaches remain restricted to the research domain.

Molecular subtypes as biomarkers of ICI response

Using molecular markers to identify subgroups of mRCC patients who experience more robust responses to ICI therapy remains a promising avenue of study.

Transcriptomic analysis in a subset of primary resected ccRCC samples obtained from patients with metastatic disease identified a 35-gene signature capable of subgrouping metastatic clear-cell renal cell carcinoma (ccmRCC) into four groups (ccRCC 1-4). These subtypes were associated with differential response to sunitinib treatment, with ccRCC 1 and 4 tumors having a lower response rate and poorer survival outcomes than ccRCC 2 and ccRCC 3 subtypes. Molecular characterization of these tumor subtypes identified unique tumor microenvironments that may explain the relative response to sunitinib treatment. For example, ccRCC 4 cancers were associated with a suppressive immune microenvironment with overexpression of PD-L1 and PD-L2, while the ccRCC 2 subgroups demonstrated a more pro-angiogenic subtype[70]. These results lead to the hypothesis that these molecular subtypes could stratify patients into groups most likely to benefit from TKI therapy, ICI monotherapy, and combined ICI therapy approaches.

This hypothesis was tested in the phase II BIONIKK trial, the first to stratify patients into treatment groups based on their molecular subtype. Patients were divided into the ccRCC risk group using the aforementioned 35-gene transcriptomic signature and were prospectively allocated into treatment groups. Patients with ccRCC 1 or 4 tumors were assigned to anti-PD1 monotherapy with nivolumab alone or anti-PD1/anti-CTLA4 with a combination of nivolumab-ipilimumab. Those patients with ccRCC 2 or ccRCC 3 group tumors received TKI therapy and a combination of nivolumab-ipilimumab. The primary outcome of this study was the overall response rate per the RECIST criteria, with survival outcomes, tolerability, and duration of the response being secondary outcomes[71]. Meylan *et al.*[72] further identified the biomarkers for the efficacy of Nivolumab (N) +/- Ipilimumab (I) in mRCC patients with TLS > 2 treated with N or NI showed a response rate of 73% and 71%, respectively. Both TLS > 2 and higher densities of Ki67/PD1 correlated with better response rates (80% *vs.* 43% $P < 0.01$) and decreased incidence of progression events (5% *vs* 36%, $P = 0.02$).

Another example of molecular subtyping in a trial setting comes from the IMmotion 150 and 151 study and the ongoing phase II OPTIC trial. The IMmotion 150 trial compared treatment with atezolizumab alone or in combination with bevacizumab to sunitinib in a cohort of 305 patients with previously untreated metastatic renal cell carcinoma. Though treatment with a combination of atezolizumab alone or in

combination with bevacizumab did not demonstrate improved PFS, the retrospective analysis did identify gene expression signatures associated with PFS involving angiogenesis, immunity (primarily T-effector presence and function), and myeloid inflammatory pathways. In particular, patients with a high angiogenesis signature score had an improved overall response rate and a longer PFS within the treatment sunitinib arm compared to those with low signature scores. Those with a high T-effector gene signature score experienced an improved overall response rate and longer PFS than those with a low score in the atezolizumab plus bevacizumab arm. In contrast, those with a high myeloid inflammation gene expression score experienced a shorter PFS in the atezolizumab plus bevacizumab and atezolizumab monotherapy treatment arms. These results suggest that such gene expression signatures may be useful tools in identifying patients more likely to benefit from various treatment regimens[73].

The findings of the IMmotion 150 study were validated and expanded upon in the phase 3 open-label IMmotion 151 study, which enrolled 915 patients and allocated them to receive either a combination of atezolizumab plus bevacizumab or sunitinib monotherapy. While this trial failed to demonstrate an overall survival benefit to combination therapy, it has provided rich biomarker data for mRCC molecular subtyping[74]. The IMmotion 151 trial retrospectively assigned patients to subgroups based on the previously identified gene expression signatures developed in the IMmotion 150 cohort. The authors again demonstrated that patients with a high angiogenesis signature score had improved PFS in the sunitinib arm, and those treated with atezolizumab plus bevacizumab had an improved PFS compared to sunitinib in subgroups with a high T-effector score or a low angiogenesis score. Based on these analyses, patients enrolled in the IMmotion 151 trial were further retrospectively classified into seven molecular subtypes - termed clusters - according to their unique genomic and transcriptomic enrichment pathways. Clusters 1 (angiogenic/stromal) and 2 (angiogenic) were enriched among the favorable risk groups as defined by the IMDC and Memorial Sloan-Kettering Cancer Center (MSKCC) scores, while clusters 4 (T-effector/proliferative), 5 (proliferative), and 6 (stromal/proliferative) were enriched among poor-risk patients.

Additionally, patients in clusters 1 and 2 demonstrated improved survival, while those in cluster 6 had poor PFS outcomes compared to other clusters, irrespective of treatment arm. Moreover, treatment with atezolizumab plus bevacizumab demonstrated improved overall response rates and longer PFS than sunitinib in clusters 4, 5, and 7 (small nucleolar RNA). Multivariable analysis of these identified molecular subtypes with clinical scores, including the MSKCC and IMDC scores, demonstrated an independent association with survival. These results suggested that these molecular subtypes may have the predictive capacity and could be incorporated with existing clinical tools to provide additional benefit, though additional prospective testing would be important prior to any clinical translation[75]. Of note, the phase II OPTIC trial is taking the first steps towards this goal, looking to utilize these molecular subgroups to prospectively assign patients to combination ICI treatment with ipilimumab plus nivolumab or ICI-TKI therapy with nivolumab and cabozantinib. Currently, in the recruitment phase, this trial will provide critical insights into the utility of molecular subtypes for treatment stratification in mRCC (NCT05361720).

Future perspectives

The above trials are a testament to the advancements in understanding the molecular underpinnings of mRCC and the role these insights can play in tailoring therapy in this patient population. Despite this progress, there are still several areas requiring further study. First, many unanswered questions remain regarding integrating these molecular subtypes with current clinical risk criteria. Further, these approaches are time-consuming and cost-prohibitive due to their reliance on multi-omic profiling. Therefore, studies on the cost-efficacy and accessibility of these tools will be critical before a transition into the clinical landscape. Additionally, molecular signature identification may benefit from targeted therapy, as there are an increasing number of TKI and PD-1/PD-L1 options available. Therefore, although these trials' results are

promising, further study is imperative to bring molecular subtypes into the clinical setting.

Clinical implication in real-world settings

Combination of lenvatinib plus everolimus for those with primary resistance

In a cohort of 7 patients that had shown resistance to VEGF-targeted TKI's or ICI therapy, a combination of lenvatinib and everolimus as either second or third-line therapy resulted in a partial response in three patients and stable disease in three patients. Progression-free survival ranged from 3 to 15 months[76].

Combination therapy as second-line therapy active in distinct clinicopathological features

This combination retrospective study included 343 patients, with 123 receiving Cabozantinib and 220 receiving Nivolumab. Patients receiving Nivolumab and first treated with Pazopanib showed a non-statistically significant median overall survival of 26.8 *vs.* 11.6 months. The OS for patients with Cabozantinib was 25.7 months as compared to Sunitinib 21.7 months, but again, not statistically significant ($P = 0.45$). Notably, Cabozantinib exhibited activity in terms of progression-free survival, particularly in patients with Clear Cell histology (7.8 *vs.* 5.4, $P = 0.026$) and those with good risk features (12.3 *vs.* 5.7, $P = 0.022$)[77].

Another phase II trial, including a high dose of Cabozantinib with atezolizumab therapy (COSMIC 021), demonstrated an encouraging clinical response[78]. However, the CONTACT-03 study did not show any promising results in patients treated after disease progression during or after immune checkpoint inhibitor therapy (either combination or monotherapy) (NCT04338269).

APPROACH TO OVERCOME ICI RESISTANCE

Targeting the TME

Colony stimulating factor 1 receptor inhibitor

M2 macrophages promote tumor neoangiogenesis, and progression, which play a role in the treatment's resistance. The expression of colony stimulating factor 1 receptor (CSF1R) allows switching the type 1 macrophages to the type II tumor associated-macrophages[79]. There are phase-1 trials currently undergoing to assess the effectiveness of combining treatment with CSF1R inhibitors and ICIs (NCT02718911, NCT02526017).

Indoleamine 2,3-dioxygenase 1 inhibitors

The indoleamine 2,3-dioxygenase 1 inhibitors deprives T cells of nutrients and can be the target for treatment. A study published in 2018 on patients with metastatic ccRCC treated with Nivolumab has shown that IDO-1 overexpression (> 10%) was found in patients with an excellent response to the treatment and thus better PFS. This study suggested that IDO could be used as a biomarker for patients with RCC[80].

A phase I/II ECHO-202/KEYNOTE 037 trial combining oral IDO-1 enzyme inhibitors with pembrolizumab was associated with a 40% objective response (8 complete and 13 with stable disease)[81]. Unfortunately, in another phase III study, IDO-1 enzyme inhibitors failed to show a positive response in patients with melanoma, so their use was stopped after the study results[82].

Stimulators of interferon genes and retinoic acid-inducible gene 1 agonists

The stimulators of interferon genes (STING)pathway promotes the production of pro-inflammatory cytokines[83]. RIG-1 stimulates natural killer cells and CD8+ T cells[84]. There are a c ouple of trials assessing STING agonist and retinoic acid-inducible gene (RIG-1) agonist use as monotherapy or in combination with ICIs (NCT03010176 and NCT 03739138)

Targeting HIF-2α

Belzutifan is a first-in-class novel therapy to inhibit HIF-2α resulting in anti-tumor activity by impairing the hypoxic signal pathway in cancer cells. A phase 1 study with patients with pre-treated ccRCC showed a 25% response rate[85]. In a phase 2 study, patients who received prior treatments (immunotherapy or chemotherapy) were treated with Belzutifan plus Cabozantinib showed an objective response rate in 16 [30.8% (95%CI: 18.7-45.1)] of 52 patients[86].

Another inhibitor of HIF is PT2385, which was studied in combination with Nivolumab in patients with mRCC who previously had received up to three treatments. It showed an objective response rate of 22% with a PFS of 10 months among patients receiving therapeutic doses of PT2385 versus 4.7 months in the sub-therapeutic group[87].

Pegylated IL-2 and cytokines

IL-2 has shown anti-tumor potential by lysing tumor cells[88,89]. In a study of patients with mRCC, high-dose IL-2 combined with Pembrolizumab has shown an objective response rate (ORR) of 69%[90]. In a study in patients with previously untreated mRCC, Bempegaldesleukin (NKTR-214), combined with Nivolumab, showed an ORR of 54% in untreated mRCC[91].

Targeting patient's intrinsic factor

Modulation of the gut microbiome

CBM58 is a bifidogenic live bacterium that can augment the effect of ICI by modulating the gut microbiome. The single-center randomized study (NCT03829111) in mRCC patients assessed nivolumab and ipilimumab with or without daily oral CBM588. The abundance of the bifidogenic bacterium was not seen. However, patients who received nivolumab-ipilimumab with CBM 588 had a significantly lower PFS (12.7 months *vs.* 2.5 months, HR 0.15, 95%CI: 0.05-0.47, $P = 0.001$) and a higher response rate (58% *vs.* 20%, $P = 0.06$) compared to those without CBM 588[92].

Recently Fecal microbiota transplantation (FMT) has been shown in several studies to augment the effect of ICI and overcome resistance, particularly in patients with melanoma[27,93,94]. Clinical trial in patients with RCC is still recruiting to assess the role of FMT in improving the efficacy of ICI (NCT04758507).

CONCLUSION

The outcomes of patients with metastatic RCC have changed significantly over the past few decades. The new therapeutic options, particularly with ICIs, have survival benefits and a durable response rate either used as a monotherapy or in combination with other therapies. However, patients may have resistance initially reflecting primary resistance or initial response to the treatment and then develop secondary resistance. Resistance to ICIs is influenced by three significant components: patient intrinsic factor, tumor cell-intrinsic factor, and contributions from the tumor microenvironment. Many innovative approaches have been studied and investigated in clinical trials to assess ICI resistance mechanisms in patients with mRCC.

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Cancer Drug Resistance

Original Article

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Statins markedly potentiate aminopeptidase inhibitor activity against (drug-resistant) human acute myeloid leukemia cells

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Abstract

Aim: This study aimed to decipher the molecular mechanism underlying the synergistic effect of inhibitors of the mevalonate-cholesterol pathway (i.e., statins) and aminopeptidase inhibitors (APis) on APi-sensitive and -resistant acute myeloid leukemia (AML) cells.

Methods: U937 cells and their sublines with low and high levels of acquired resistance to (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester (CHR2863), an APi prodrug, served as main AML cell line models. Drug combination effects were assessed

with CHR2863 and *in vitro* non-toxic concentrations of various statins upon cell growth inhibition, cell cycle effects, and apoptosis induction. Mechanistic studies involved analysis of Rheb prenylation required for mTOR activation.

Results: A strong synergy of CHR2863 with the statins simvastatin, fluvastatin, lovastatin, and pravastatin was demonstrated in U937 cells and two CHR2863-resistant sublines. This potent synergy between simvastatin and CHR2863 was also observed with a series of other human AML cell lines (e.g., THP1, MV4-11, and KG1), but not with acute lymphocytic leukemia or multiple solid tumor cell lines. This synergistic activity was: (i) specific for APis (e.g., CHR2863 and Bestatin), rather than for other cytotoxic agents; and (ii) corroborated by enhanced induction of apoptosis and cell cycle arrest which increased the sub-G1 fraction. Consistently, statin potentiation of CHR2863 activity was abrogated by co-administration of mevalonate and/or farnesyl pyrophosphate, suggesting the involvement of protein prenylation; this was experimentally confirmed by impaired Rheb prenylation by simvastatin.

Conclusion: These novel findings suggest that the combined inhibitory effect of impaired Rheb prenylation and CHR2863-dependent mTOR inhibition instigates a potent synergistic inhibition of statins and APis on human AML cells.

Keywords: Aminopeptidase, statins, mevalonate pathway, carboxyl esterase, Rheb, mTOR

INTRODUCTION

Targeting of protein degradation pathways has provided new therapeutic opportunities for hematological malignancies. Proteasome inhibitors, with Bortezomib (Velcade®) as a prototypical representative, gained an established place in chemotherapeutic treatment regimens of multiple myeloma[1,2]. Aminopeptidases operating downstream of the proteasome were also identified as druggable targets, with Bestatin as the founding drug displaying activity mainly against solid tumors[3,4]. Tosedostat represents a next-generation aminopeptidase inhibitor (APi) that displays activity as monotherapy as well as in combination with various chemotherapeutic drugs, including cytarabine, daunorubicin and histone deacetylase (HDAC) inhibitors[5-9]. Moreover, Tosedostat demonstrated clinical activity in phase I-III combination chemotherapy for acute myeloid leukemia (AML)[6,10-18] and multiple myeloma (MM)[19], and has been evaluated for the treatment of solid tumors[20,21]. Tosedostat, and a close structural analogue (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester (CHR2863), are APi prodrugs with an esterase-sensitive motif[5]. As hydrophobic drugs, they can freely diffuse into cells wherein they are converted by intracellular esterases to their hydrophilic acid active metabolites that enhance their cellular retention and promote inhibition of multiple aminopeptidases. The latter provokes an amino acid deprivation response, inhibition of mTOR activity, and blockade of protein synthesis[5]. Recently, we demonstrated[22] that the cytotoxic activity of CHR2863 against U937 myeloid cells relies on carboxylesterase 1 (CES1) activity. Consistently, down-regulation of CES1 and loss of CHR2863 conversion to its hydrophilic active metabolite constituted a dominant mechanism of acquired resistance to CHR2863[22]. CES1 has an essential physiological function in cholesterol metabolism by converting cholesteryl esters to cholesterol[23,24]. Since AML cells harbor an aberrant cholesterol metabolism[25-27], we explored whether or not statins as inhibitors of the mevalonate-cholesterol biosynthetic pathway potentiate the cytotoxic activity of APi (pro) drugs. In earlier studies, statins displayed differential sensitization of AML cells[28-32] but were also able to enhance the sensitivity of various anti-leukemic drugs, including cytarabine, daunorubicin and the cell cycle inhibitor UCN-01[33,34]. Here, we discovered that various statins markedly potentiated the cytotoxic activity of CHR2863 in multiple human AML cell lines as well as in CHR2863-resistant cells, by a mechanism that involves perturbation of Rheb prenylation as an essential complementary factor to mTOR inhibition by APis. These novel findings uncover a potent therapeutic combination of statins and APis which may warrant a further clinical evaluation in AML treatment.

METHODS

Chemicals

Simvastatin (430-104-M) was obtained from Alexis Biochemicals (San Diego, CA USA). Fluvastatin (10010337) and lovastatin (10010338) were purchased from Cayman Chemical Co (Ann Arbor, MI, USA). Pravastatin (P4498), R-mevalonic acid (50838), squalene (S3632), farnesyl pyrophosphate (F6892), geranylgeranyl pyrophosphate (G6025), FTI-277 (F9803), bestatin (B8385) and daunorubicin (30450) were from Sigma Chemical Co (St. Louis MO, USA). Bortezomib was obtained from the VUmc Pharmacy department. CHR2863, (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid (CHR6768), and (S)-[3-(7-Hydroxycarbamoyl-heptanoylamino)-benzylamino-phenyl acetic acid cyclopentyl ester (CHR2875)[5,35] were provided by Dr. A. Drummond (Chroma Pharmaceuticals Ltd, Abingdon, UK) and dissolved in dimethyl sulfoxide as 10 mM stock solutions and stored at -20 °C.

Antibodies

The following antibodies were used for Western blot analysis: CES1 monoclonal antibody (Lifespan Biosciences, Seattle, WA, USA, LS-C498701, 1:1,000 dilution) and rabbit polyclonal antibodies at 1:1000 dilutions: total Akt (#9272), phospho-Akt (Ser308) (C31E5E) (#2965), phospho-Akt (Ser473) (#9271), total mTOR (7C10) (#2983), phospho-mTOR (Ser2448) (#2971), phospho-mTOR (Ser2481) (#2974), total S6K (#9202), phospho-S6Kp70 (Th389) (#9205) and Rheb (#4935) all from Cell Signalling Technology, Danvers, MA, USA. β-Actin antibody was from Sigma-Aldrich, St. Louis, MO, USA (A2172, 1:10,000 dilutions). Secondary antibodies included goat anti-mouse or goat anti-rabbit antibodies conjugated to IRDye®800CW (1:10.000, Odyssey; LI-COR, Biosciences, Nebraska, USA).

Cell culture

Human U937 myelomonocytic leukemia cells (ATCC, Manassas, VA, USA) and 2 sublines U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} (resistant to 0.2 and 5μM CHR2863, respectively and characterized by 14- and 270-fold acquired resistance to CHR2863) were isolated and cultured as described previously[22]. Other human myeloid leukemia cell lines (THP1, MV4-11, and KG1), human lymphoblastic cell lines (CEM and CEM/Vbl), human ovarian carcinoma cell lines (2008 and 2008/MRP1), human breast carcinoma cell lines (MCF7/WT and MCF7/MR), human lung cancer (SW1573) and human nasopharyngeal carcinoma cells (KB) were cultured as described previously[36-41]. Briefly, cells were grown in RPMI-1640 medium (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS, PAA Cell Culture Company, Pasching, Austria), 20 mM HEPES, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (all from Lonza, Verviers, Belgium). The cell lines were cultured in 25 cm^2 culture flasks (Greiner Bio-One GmbH, Frickenhansen, Germany) in 10 mL medium at an initial density of 3 × 10^5 cells/mL (or 1.25 × 10^4/cm^2 for adherent cells) and in a humidified atmosphere at 37 °C and 5% CO_2. Cell cultures were passaged every 3-4 days. Cells were regularly checked, and found negative, for mycoplasma contamination.

Western blotting

Western blot analysis was performed essentially as described by Verbrugge et al.[22]. Briefly, cell lysates were prepared from 5 × 10^6 cells suspended in 150 μL ice-cold lysis buffer (Cell Signalling Technology, #9803) containing 4% Protease Inhibitor Cocktail (PIC) and 1 mM $NaVO_4$. Supernatant fractions were collected by centrifugation (13,000 × g for 10 min, 4 °C), and 30 μg protein aliquots were resolved on a 4%-20% TGX pre-cast SDS PAGE gels (Bio-Rad), followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) suitable for chemiluminescent detection by the Odyssey Infrared Imaging System (PerkinElmer, Zaventem, Belgium). The membranes were pre-incubated in blocking buffer (Odyssey Blocking Buffer, LI-COR, Biosciences, Nebraska, USA) for 1 hr. Next, membranes were incubated

overnight (4 °C) with primary antibodies and β-actin for control of equal loading. After three washing steps (PBS/0.05% Tween20), the membranes were incubated (1 hr) with secondary antibodies, followed by antibody detection with the LI-COR Odyssey scanner (Biosciences) and digital image acquisition/quantification with the Odyssey infrared imaging system software (version 3.0.16, LI-COR Biosciences) according to the manufacturer's instructions.

Apoptosis assay

Cells were collected and washed three times with ice-cold PBS. Early phase apoptosis was determined by the Annexin-V/7AAD Kit (PN IM3614, Beckman Coulter) using a FACSCalibur flow cytometer (Becton and Dickinson, San Jose, CA) using the manufacturer's protocol. Briefly, cells were washed and resuspended in binding buffer. Annexin-V (1:10) and 7-Amino-Actinomycin (7AAD, 1:20) were added and incubated for 15 min on ice in the dark. Binding buffer was added and analyzed by flow cytometry followed within 1 hr. Annexin-V-positive and 7AAD-positive cells were considered as apoptotic cells.

Cell cycle analysis

Cell cycle analysis was performed using a FACSCalibur flow cytometer and propidium Iodide (PI) staining[42]. Cells were washed three times with ice-cold PBS and resuspended in medium. PI (5% Propidium Iodide dissolved in PBS with 1% trisodium citrate, 0.1% RNAse and 0.1% Triton X-100) was added, and cells were vortexed and measured directly by flow cytometry. Fluorescence signal was detected through the FL2 channel. FACS analysis was performed using Cell Quest software.

Miscellaneous assays

Quantitative RT-PCR analysis to assess CES1 mRNA levels and LC-MS/MS analyses to determine the conversion of the prodrug CHR2863 to its metabolite CHR6768 were performed essentially as described before[22].

Statistical analysis of synergism

Combination indices (CI) for analysis of synergism between simvastatin and CHR2863 were calculated by CalcuSyn software (Version 1.1.1, copyright Biosoft 1996)[43] and the multiplicative model to predict the effect of drug combinations[44].

Statistics

A two-tailed paired Student's t-test was used for comparison between groups. Significant differences were defined at $P < 0.05$.

RESULTS

Simvastatin synergizes CHR2863 growth inhibition in parental and CHR2863-resistant U937 cells

Growth inhibitory effects of CHR2863 were determined in human U937/WT cells and two variants, one low (U937/CHR2863$^{R0.2}$ and one highly (U937/CHR2863^{R5}) CHR2863-resistant U937 cells[22] in the absence or presence of a maximal *in vitro* non-toxic concentration of 2.0-2.5 μM simvastatin [Figure 1]. For U937/WT cells [Figure 1A], simvastatin potentiated the growth inhibitory effects of CHR2863 by 14-fold (from IC_{50}: 60.9 ± 15.8 nM to 4.3 ± 1.3 nM). Consistently, simvastatin potentiated CHR2863 activity 18-fold in U937/CHR2863$^{R0.2}$ cells (from IC_{50}: 682 ± 182 nM to an IC_{50} of 37.8 ± 10.8 nM), which compares to the sensitivity of U937/WT cells to CHR2863 [Figure 1B]. Lastly, simvastatin also potentiated the growth inhibitory effect of CHR2863 in U937/CHR2863^{R5} cells, albeit with a lower potentiation factor, 3.3-fold (from IC_{50}: 12,900 ± 4,300 nM to 3,900 ± 2,200 nM) [Figure 1C]. Analysis of the dose-response effect of drug interactions at a constant dose of simvastatin and fractional effect by CHR2863 revealed remarkable combination indices (CI) well below 1 for parental and CHR2863-resistant U937 cells, indicating a strong

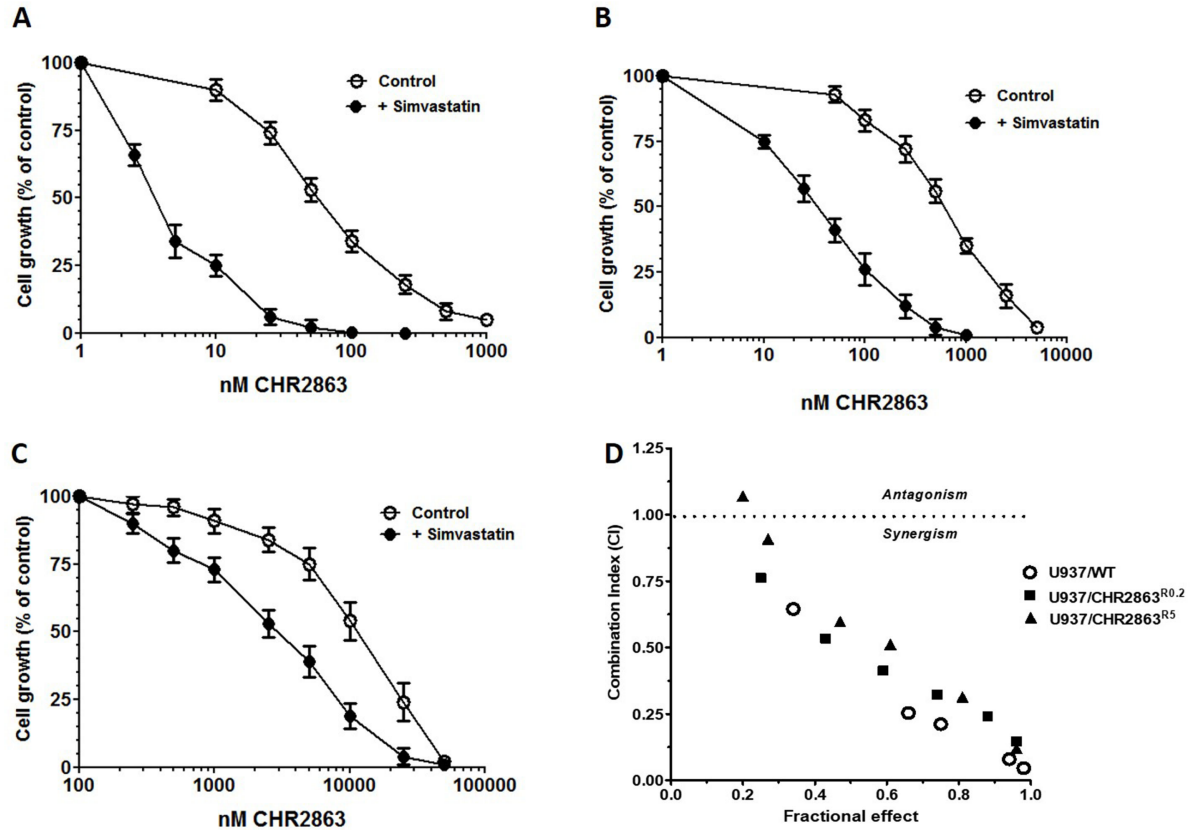

Figure 1. Growth inhibitory effects of CHR2863 for (A) U937/WT, (B) U937/CHR2863$^{R0.2}$ and (C) U937/CHR2863^{R5} cells in the absence and presence of maximal non-toxic concentrations of simvastatin (2 μM, 2.5 μM, and 2.5 μM, respectively). Cell growth inhibition was determined after 72 h of drug exposure. The results depicted are the mean ± SE of 6-10 separate experiments; (D) Combination index - fraction affected plot from (A-C) of the combination simvastatin (fixed concentration) and CHR2863 for U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

synergistic interaction [Figure 1D], especially at the FA > 0.5, which is considered as relevant because growth is almost completely inhibited.

Multiple statins synergize with CHR2863 in U937/WT and CHR2863-resistant U937 cells

We next assessed whether statins other than simvastatin also have the ability to synergize with CHR2863 activity in U937/WT and CHR2863-resistant cells. Maximal *in vitro* non-toxic concentrations of the naturally-derived statins lovastatin (2.5-5 μM) and pravastatin (100-200 μM), as well as the synthetic statin fluvastatin (0.5-1 μM) exhibited comparable capacities as simvastatin to potentiate CHR2863 activity as revealed by their potentiation factors (ratio IC_{50} CHR2863 with statin over IC_{50} CHR2863 without statin) [Figure 2].

Statin potentiation is selective for aminopeptidase inhibitors

To assess whether statin potentiation of the APi prodrug CHR2863 also occurs with a direct APi, we tested whether the growth inhibition by bestatin[3,45] is potentiated by simvastatin. Indeed, simvastatin potentiated both CHR2863 and bestatin activities with similar potentiation factors in U937/WT and CHR2863-resistant U937 cells [Figure 3 and Supplementary Figure 1]. Moreover, statin potentiation appeared selective for APis as no potentiation was observed for two types of other drugs: CHR2875, an HDAC inhibitor prodrug[35],

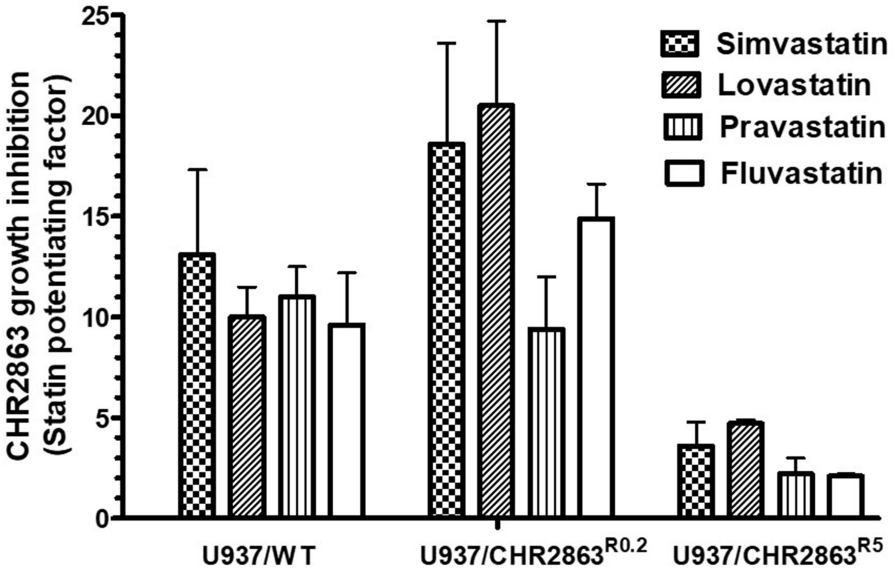

Figure 2. The potentiating effect of maximal non-toxic concentrations of various statins on the CHR2863 activity in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. The concentrations of simvastatin, lovastatin, pravastatin and fluvastatin were 2 μM, 2.5 μM, 100 μM and 0.5 μM, respectively, for U937/WT cells, and 2.5 μM, 5 μM, 200 μM and 1 μM, respectively, for U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. CHR2863 dose response curves in combination with statins were generated over a CHR2863 concentration range of 0-1 μM for U937/WT cells, 0-5 μM for U937/CHR2863$^{R0.2}$ cells and 0-50 μM CHR2863 for U937/CHR2863^{R5} cells, essentially as shown in Figure 1. Statin potentiating factor is defined as the ratio of IC_{50} (50% growth inhibition) of cell culture without statins *vs.* IC_{50} of cell cultures in the presence of statins. Cell growth inhibition was determined after 72 h of drug exposure. The results depicted are the mean ± SD of 3-4 independent experiments. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

which is bioactivated similarly as CHR2863, and daunorubicin evaluated in combination chemotherapy with Tosedostat for AML [Figure 3 and Supplementary Figure 1].

Statin potentiation of CHR2863 activity is primarily restricted to AML cells

To determine whether statin potentiation of CHR2863 activity occurs in various human AML cells other than U937 cells, the potentiating effect of maximal non-toxic concentrations of simvastatin was examined in multiple AML cell lines, acute lymphocytic leukemia (ALL) CCRF-CEM cells as well as a panel of (multidrug resistance-related) solid tumor cell lines [Figure 4 and Supplementary Figure 2]. As with U937 cells, CHR2863 growth inhibition was significantly potentiated by simvastatin in various AML cell lines, including THP1, MV4-11 and, to a lower extent, KG1 cells. In contrast, simvastatin had no potentiating effect in CCRF-CEM cells and a P-glycoprotein/MDR1-overexpressing subline CEM/VBL, although it should be emphasized that these cells had a low intrinsic sensitivity to CHR2863 (IC_{50} > 10 μM). The panel of solid tumor cell lines displayed variable sensitivity to CHR2863 (IC_{50}: 0.13-6.7 μM); with the exception of MCF7/MR cells, none showed a potentiating effect by simvastatin. These results indicate that the simvastatin potentiating effect of CHR2863 is largely restricted to AML cells.

Simvastatin - CHR2863 combinations: impact on cell growth, apoptosis and cell cycle

An exposure of 48 h to maximal *in vitro* non-toxic concentrations of simvastatin and minimally cytotoxic (≈ IC_{10}) concentrations of CHR2863 was tested for the impact on cell viability, apoptosis induction and sub-G_1 fraction/cell cycle distribution of U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells [Figure 5].

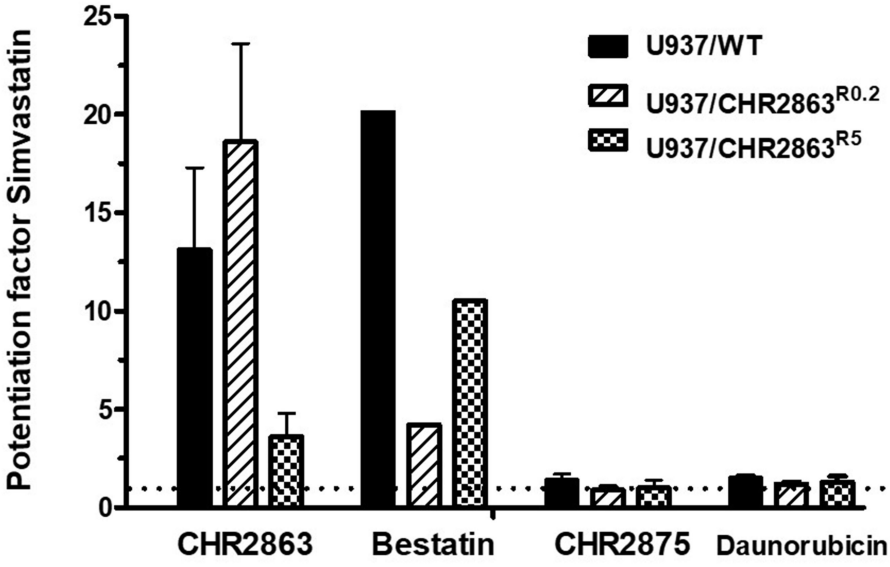

Figure 3. Selectivity of simvastatin-potentiating effect for APis. Effect of non-toxic concentrations of simvastatin (2-2.5 μM) on the growth inhibitory activity of the APis CHR2863 and bestatin, HDAC inhibitor prodrug CHR2875, and daunorubicin in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. Simvastatin potentiation factor is defined as the ratio of IC_{50} (50% growth inhibition) of cell culture without statins *vs.* IC_{50} of cell cultures in the presence of statins. Cell growth inhibition was determined after 72 h of drug exposure. Results depicted are the mean of two separate experiments (for bestatin) and the mean ± SD of 3-4 separate experiments for CHR2863, CHR2875 and daunorubicin. IC50 values of U937/WT, U937/CHR2863R0.2 and U937/CHR2863R5 cells for CHR2863 are: 52 ± 16 nM, 713 ± 212 nM, and 14,047 ± 5,521 nM, respectively; for Bestatin: 158 ± 15 μM, 169 ± 32 μM, and 177 ± 14 μM, respectively; for CHR2875: 158 ± 9 nM, 86 ± 13 nM, and 147 ± 36 nM, respectively; and for daunorubicin: 16 ± 1 nM, 16 ± 2 nM, and 15 ± 3 nM, respectively. APis: aminopeptidase inhibitors; CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

Bortezomib (0.1 μM) and a high concentration of CHR2863 (6 μM) were included as a reference control. Single doses of CHR2863 and simvastatin had no effect on cell viability, whereas their combination significantly reduced cell viability in all three cell lines [Figure 5A], which was accompanied by a significantly increased apoptosis [Figure 5B and Supplementary Figure 3A] and an increase in the sub-G_1 fraction [Figure 5C and Supplementary Figure 3B]. No visible alterations in cell cycle distribution were noted at the tested concentrations of CHR2863, simvastatin or their combination [Figure 5D]. The impact of simvastatin and CHR2863 combinations on cell viability and apoptosis for the U937 cell lines were also found for three other AML cell lines; THP1 and MV4-11 and to a lesser extent for KG1 cells [Supplementary Figure 3C].

Reversal of simvastatin potentiation of CHR2863 activity by mevalonic acid, farnesyl pyrophosphate and geranylgeranyl pyrophosphate

To determine whether or not the statin-induced inhibition of HMG-CoA reductase is implicated in the simvastatin potentiation of CHR2863 cytotoxicity, we assessed whether or not intermediates of the mevalonate pathway, i.e., mevalonic acid (MVA), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) were able to abrogate the potentiating effect of simvastatin. Increasing concentrations of MVA fully abrogated simvastatin potentiation of CHR2863 growth inhibition in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells [Figure 6A]. Likewise, increasing concentrations of FPP also abrogated the simvastatin potentiation effect of CHR2863 in U937/WT and U937/CHR2863$^{R0.2}$ cells, albeit to a slightly lower extent than MVA [Figure 6B]. Of note, FPP failed to abrogate the simvastatin

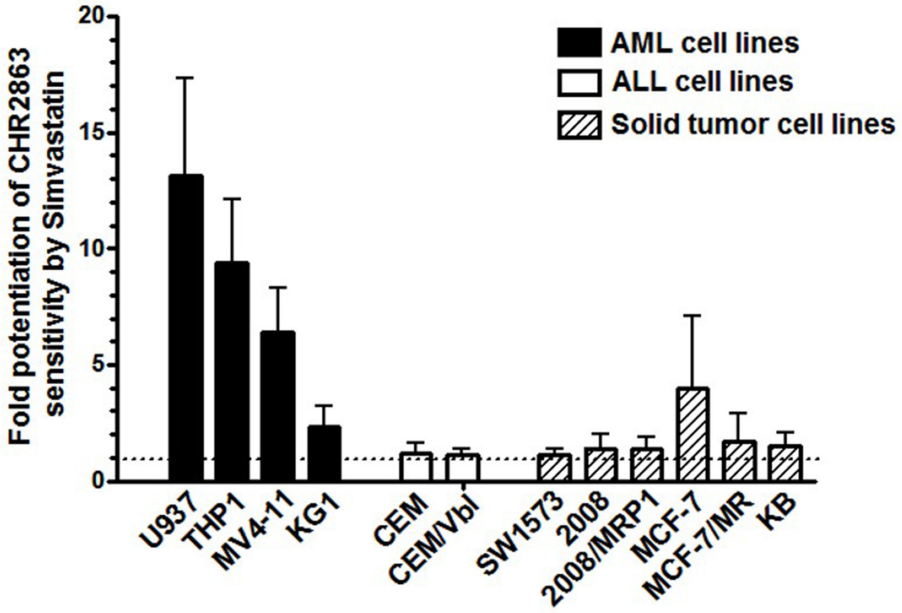

Figure 4. Simvastatin potentiation of CHR2863 activity in human AML cell lines *vs.* human lymphoid and solid tumor cell lines. Cell growth inhibition was determined after 72 h of drug exposure in the absence or presence of maximal non-toxic concentrations of simvastatin, being (between brackets) for: U937 (2 μM), THP1 (2.5 μM), MV4-11 (2.5 μM), KG1 (10 μM), CCRF-CEM (2.5 μM), CEM/Vbl (2.5 μM), SW1573 (0.2 μM), 2008 (0.75 μM), 2008/MRP1 (2.5 μM), MCF7 (1 μM), MCF7/MR (2.5 μM) and KB (1 μM). Simvastatin potentiation factor is defined as the ratio of IC_{50} (50% growth inhibition) of cell culture without statins *vs.* IC_{50} of cell cultures in the presence of statins. IC_{50} values (between brackets) for CHR2863 for the various cell lines (in the absence of simvastatin) were: U937 (61 ± 16 nM), THP1 (1172 ± 807 nM), MV4-11 (282 ± 51 nM), KG1 (394 ± 144 nM), CCRF-CEM (11,170 ± 5,100 nM), CEM/Vbl (29,100 ± 5,900 nM), SW1573 (6,625 ± 3,020 nM), 2008 (2,020 ± 1,080 nM), 2008/MRP1 (6,700 ± 2,560 nM), MCF7 (453 ± 400 nM), MCF7/MR (386 ± 64 nM), and KB (132 ± 50 nM). The results depicted are the mean ± SD of 3-5 independent experiments. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

potentiation effect of CHR2863 in U937/CHR2863^{R5} cells [Figure 6B]. Lastly, GGPP abrogated the simvastatin potentiation effect of CHR2863 in U937/WT and U937/CHR2863$^{R0.2}$ cells at an optimal concentration of 0.1 μM; above this concentration, the abrogating effect was lost [Figure 6C]. GGPP was also unable to abrogate the potentiation effect of simvastatin in U937/CHR2863^{R5} cells [Figure 6C].

Given the variable effects of FPP in abrogating the potentiating effect of simvastatin of CHR2863 in U937/WT and U937/CHR2863$^{R0.2}$ cells *vs.* U937/CHR2863^{R5} cells, we further examined whether a farnesyltransferase inhibitor (FTI-277) had similar effects on CHR2863 potentiation in these cells. Combinations of FTI-277 and CHR2863 were synergistic in U937/WT and additive in U937/CHR2863$^{R0.2}$ cells, whereas U937/CHR2863^{R5} cells were resistant to FTI-277 and no potentiation was found [Supplementary Figure 4].

Simvastatin potentiation of CHR2863: mechanistic studies

To explore the mechanistic basis underlying the potentiation of CHR2863 growth inhibition by simvastatin, we first examined whether simvastatin upregulated the expression of carboxylesterase 1 (CES1), the enzyme mediating the conversion of CHR2863 to its active metabolite CHR6768[22]. Western blot analysis revealed that CES1 expression (as well as its other family members CES2 and CES3) in U937/WT, U937/CHR2863$^{R0.2}$, and U937/CHR2863^{R5} cells was not altered by simvastatin and CHR2863 alone, in combination, and in combination with MVA [Supplementary Figure 5]. Notably, U937/CHR2863^{R5} cells

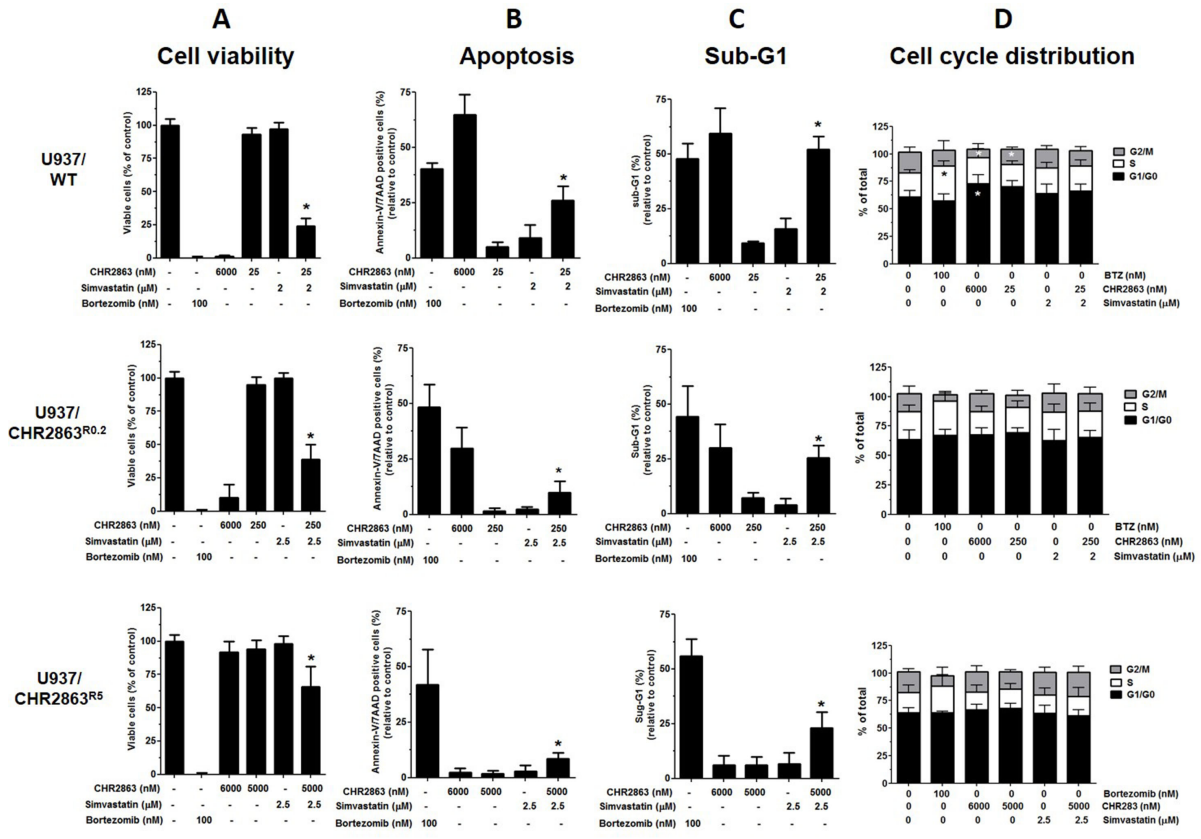

Figure 5. Effect of simvastatin and CHR2863 combinations on cell viability, apoptosis induction and cell cycle distribution in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. Simvastatin concentrations used for U937/WT cells, U937/CHR2863R0.2 and U937/CHR2863R5 cells were maximal in vitro non-toxic concentrations: 2 μM, 2.5 μM and 2.5 μM, respectively. For CHR2863, minimally cytotoxic (≈ IC10) were selected (from Figure 1), i.e., 25 nM, 250 nM and 5 μM for U937/WT cells, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells, respectively. Cells (3×10^5/mL in 10 mL medium) were incubated for 48 h with the indicated concentrations of simvastatin, CHR2863 and their combination and assessed for the impact on (A) cell viability, (B) apoptosis induction, (C) sub-G1 fraction and (D) cell cycle distribution. Cells incubated for 24 h with bortezomib or 48 h with 6 μM CHR2863 served as a control for cell growth inhibition and apoptosis induction. Percentages of apoptotic cells in control untreated U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells were 4.6% ± 1.9%, 5.2% ± 1.2% and 6.1% ± 0.9%, respectively. Sub-G1 fractions in control untreated U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells were 4.2% ± 2.0%, 9.1% ± 6.5% and 8.2% ± 2.2%, respectively. The results depicted are the mean ± SD of 4-5 independent experiments. *Combination statistically significant ($P < 0.05$) different compared to single drugs control cells. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

displayed markedly decreased CES1 expression levels as shown earlier[22]. Consistent with unaltered CES1 expression levels in the presence of simvastatin, the ability of U937/WT and U937/CHR2863$^{R0.2}$ cells to enzymatically convert CHR2863 to its active metabolite CHR6768 was unchanged, while U937/CHR2863^{R5} cells had lower levels in line with their lack of CES1 activity [Supplementary Figure 6].

Aminopeptidase inhibition can regulate mTOR activity[5]. Therefore, we evaluated whether concentrations of simvastatin and CHR2863 which showed an enhanced growth inhibitory effect (after 48 hr drug incubation) were also associated with an altered cellular phosphorylation status of intermediates of the ERK/Akt/mTOR pathway. Analysis of pERK(Thr202/Tyr204), pAkt(Ser473), pmTOR(Ser2448), pmTOR(Ser2481) and pS6Kp70(Th389) levels in U937/WT, U937/CHR2863(200), and U937/CHR2863(5μM) cells showed no major differences upon exposure to CHR2863, simvastatin, MVA, and their combinations [Supplementary Figure 7]. This suggests that other mechanisms play a prominent

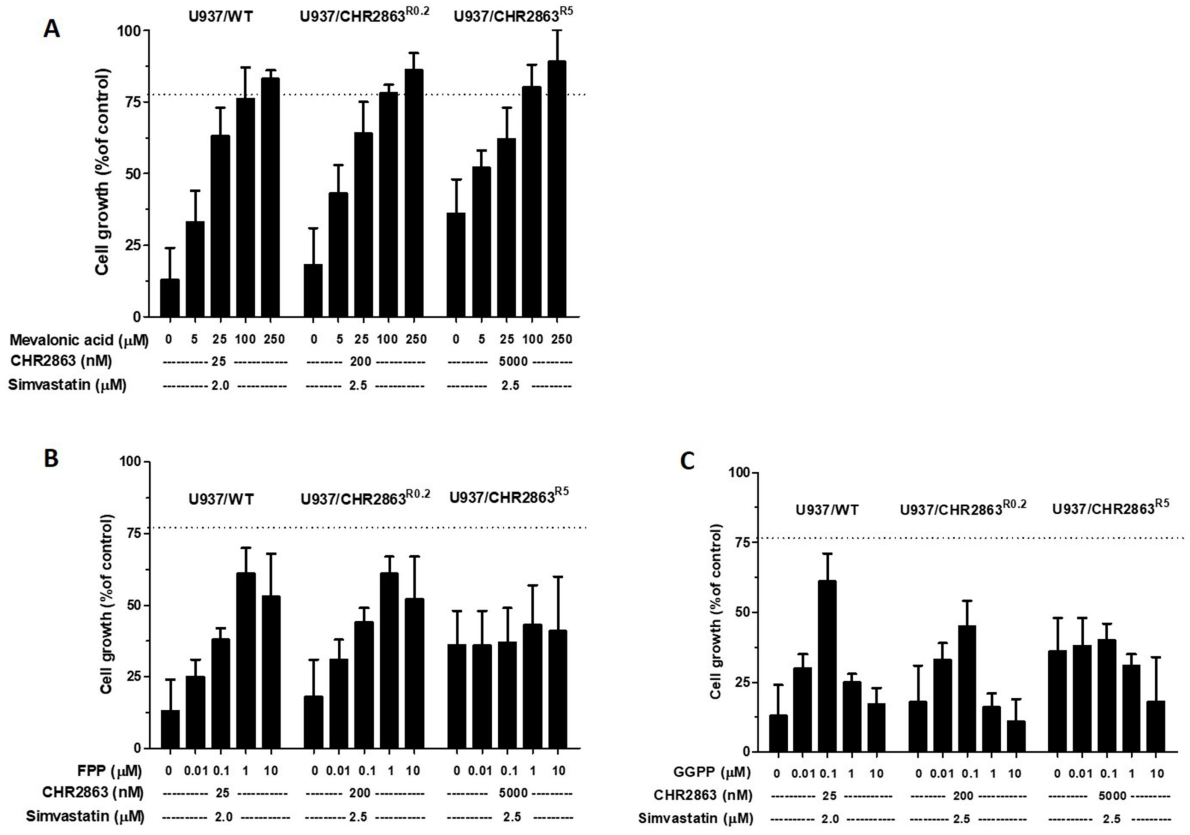

Figure 6. Effects of mevalonic acid, farnesyl pyrophosphate and geranylgeranyl pyrophosphate on simvastatin potentiation of CHR2863 activity. U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells were incubated for 72 h with the indicated concentrations of CHR2863 and non-toxic concentration of simvastatin in the presence of increasing concentrations of (A) mevalonic acid, (B) farnesyl pyrophosphate (FPP) and (C) geranylgeranyl pyrophosphate (GGPP). Results, presented as cell growth relative to control, are the mean ± SD of 4 independent experiments. The dashed line indicates the mean growth inhibition by CHR2863 alone. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

role in the synergy between statins and CHR2863.

Statins are known to impair the prenylation and thus membrane localization of various proteins[46-48]. In the context of mTOR activation, it has been demonstrated that lysosomal membrane integration of Rheb protein is of relevance and is prenylation-dependent[49-51]. To this end, we examined whether under conditions that potentiated CHR2863 activity, simvastatin interfered with Rheb prenylation in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells. Indeed, exposure to simvastatin resulted in a marked increase in unprenylated Rheb in all three tumor cell lines [Figure 7], as did the exposure to FTI-277. The level of unprenylated Rheb was maintained in CHR2863 + simvastatin combinations, whereas exposure to CHR2863 alone had no effect on Rheb prenylation status. MVA and FPP, but not GGPP, abrogated the unprenylation impact of simvastatin alone and in combination with CHR2863. Hence, this profile of Rheb unprenylation parallels the statin and inhibitor FTI-277-induced potentiation of CHR2863 activity in U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells.

A composite summary model which proposes a mechanistic basis for the synergistic action of APIs and statins in AML cells is presented and discussed in [Figure 8].

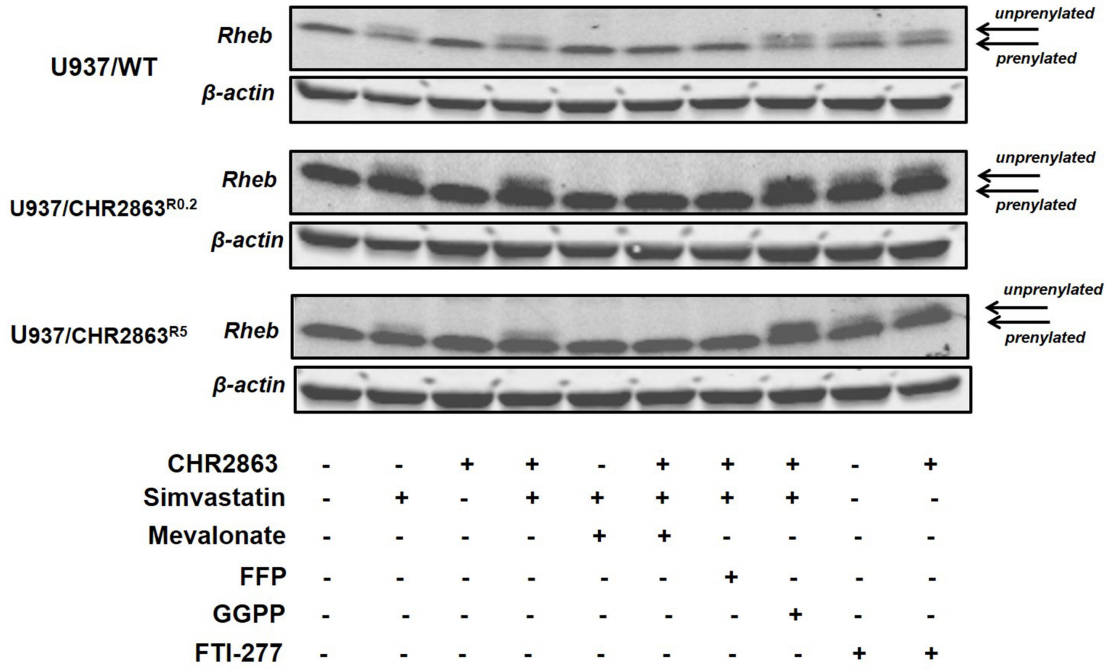

Figure 7. Effect of simvastatin and CHR2863 combinations on Rheb prenylation. U937/WT, U937/CHR2863$^{R0.2}$ and U937/CHR2863^{R5} cells were incubated for 48 h with simvastatin, CHR2863, and their combination (as described in Figure 5), with or without the addition of MVA (100 μM), FPP (2 μM), GGPP (1 μM) or FTI-277 (10 μM). The slower (upper) migrating band represents unprenylated Rheb, and the faster (lower) migrating band represents prenylated Rheb. CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

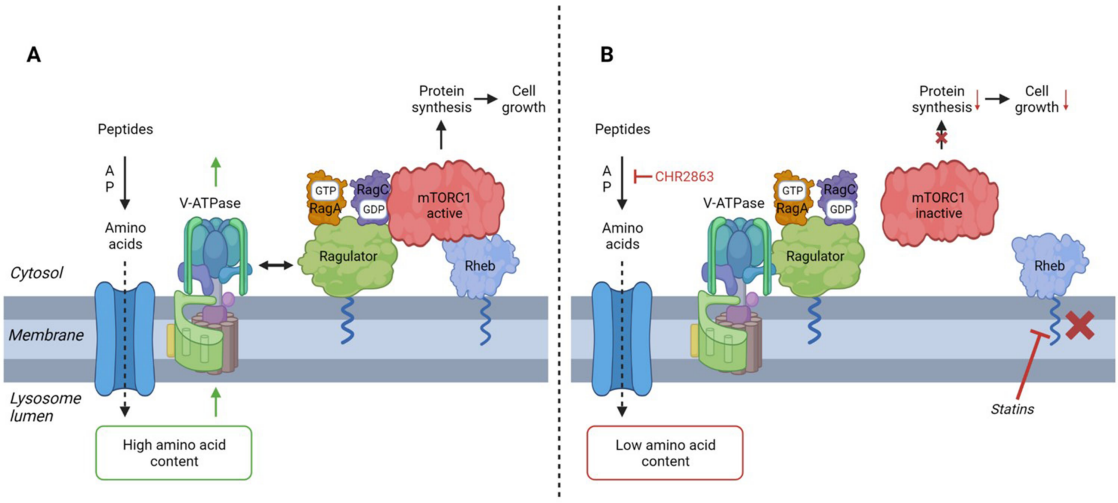

Figure 8. Proposed model for synergistic action of statins and APi CHR2863. (A) Peptide breakdown by aminopeptidases provides amino acids for re-utilization in protein synthesis. According to previously described models[49-51,56,62,63,66], an increased intralysomal amino acid content triggers dissociation of V-ATPase and Ragulator-Rag-mTORC1 complex. Binding of the latter complex to (prenylated) Rheb (in the lysosomal membrane) and membrane association of Ragulator will then induce mTOR activation and initiation of protein synthesis; (B) Inhibition of aminopeptidases by CHR2863 (or bestatin) will reduce the intralysomal amino acid content and dissociation of the Ragulator-Rag complex from mTORC1. By a different mechanism, statins may block Rheb prenylation and abolish its lysosomal membrane localization. The combined effect of CHR2863 and statins may then synergize in impairing mTOR activation, protein synthesis and inhibiting cell growth. The figure was created via *BioRender*. APi: Aminopeptidase inhibitor; CHR2863: (6S)-[(R)-2-((S)-Hydroxy-hydroxycarbamoyl-methoxy-methyl)-4-methyl-pentanoylamino]-3,3 dimethyl-butyric acid cyclopentyl ester.

DISCUSSION

Aberrant cholesterol metabolism is a characteristic feature of AML cells and has been exploited for therapeutic interventions with statins as inhibitors of HMG-CoA reductase, the key enzyme in the MVA-cholesterol pathway[26,46,52]. Both *in vitro* and *in vivo* studies demonstrated that high concentrations of statins can induce apoptosis in AML cells through perturbations of prenylation and membrane anchoring of proteins involved in signal transduction pathways. These include disruption of Ras family members and pro-survival pathways such as MEK/ERK and PI3K/Akt/mTOR[29-31,53-56]. Furthermore, statins also elicit additive/synergistic effects with various other chemotherapeutic drugs[33,34,57]. The current study is the first report to reveal that non-toxic concentrations of statins markedly potentiate the growth inhibitory effects of either a prodrug (CHR2863) or a direct inhibitor (bestatin) of aminopeptidases in human AML cells. Hence, these findings bear important implications for future therapeutics as well as overcoming chemoresistance in AML.

APis such as Tosedostat and its close structural analogue CHR2863 are prodrugs that rely on esterase activities for their conversion to active metabolites that can inhibit multiple aminopeptidases, thereby provoking amino acid depletion[5]. Earlier, we demonstrated that CES1 is the most likely candidate enzyme for the bio-activation of these prodrugs, given the high CES1 expression in myeloid cell lines and M4 and M5 FAB subtypes of AML clinical specimens[22]. The role of CES1 in this enzymatic bio-activation was further substantiated by the fact that acquired resistance to CHR2863 in AML cells was mediated by downregulation of CES1 expression. Regarding CHR2863 resistance, combinations of *in vitro* non-toxic concentrations of statins were able to sensitize 14-fold resistant U937/CHR2863$^{R0.2}$ cells, hence restoring WT sensitivity. Highly (270-fold) CHR2863 resistant U937/CHR2863^{R5} cells could also be sensitized by co-administration of statins, albeit to a lower extent (3-4 fold), even given the fact that active metabolite formation was almost 100-fold lower than in WT cells. Statin-dependent sensitization of CHR2863-resistant cells did not involve increased CES1 expression and/or enhanced active metabolite formation, suggesting that other mechanisms account for this potentiation effect. In drug combination experiments, the combination of simvastatin and CHR2863 led to a significant enhancement of apoptosis induction as reflected in the high accumulation of cells in sub-G_1 fraction, whereas treatment with either drug alone had a minimal effect. Beyond apoptosis, it cannot be ruled out that alternative mechanisms, e.g., ferroptosis[58,59], contribute to cell death. Furthermore, cell cycle analysis of simvastatin + CHR2863 combinations did not reveal any distinct cell cycle arrest in G_1/G_0-, S- or G2/M-phase, suggesting that the drug treatment did not interfere with specific cell cycle phases or checkpoints.

Further experiments with intermediates of the MVA-cholesterol pathway, including FPP, GGPP and MVA, were performed to identify the mechanism underlying statin-dependent potentiation of CHR2863 activity. Apoptosis induced by statins in AML cells was reversed by the addition of MVA and GGPP rather than FFP[28]. With respect to simvastatin potentiation of CHR2863 activity in U937 cells, GGPP reversed the potentiation effect in a narrow concentration range around 100 nM; above this concentration, the reversal effect was lost. The mechanistic reason for this decline in reversal effect beyond 100 nM GGPP is unclear and warrants further studies. The full reversal was observed with increasing concentrations of FPP and MVA, suggesting that perturbations in protein farnesylation are involved in the potentiation effect. Interestingly though, highly CHR2863-resistant U937/CHR2863^{R5} cells were unresponsive to FPP, which could be consistent with their refractoriness to the farnesyltransferase inhibitor FTI-277 [Supplementary Figure 4]. In parental U937/WT cells, synergistic growth inhibitory effects of FTI-277 and CHR2863 combinations mimicked the simvastatin-CHR2863 combinations; however, upon the acquisition of CHR2863 resistance, the potentiation effect of FTI-277 on CHR2863 activity was gradually lost. Ding *et al.* showed that acquired resistance of U937 cells to another FTI, tipifarnib, involved alterations in Rheb

prenylation and loss of inhibition of Rheb-induced mTOR signaling[60]. Consistently, the acquisition of CHR2863 resistance was also shown to be accompanied by activation of the Akt/mTOR pro-survival pathway, as reflected by a marked gain of sensitivity to the mTOR inhibitor rapamycin[22]. Given that Rheb prenylation is required for mTOR activation[56,61-66], loss of prenylation through the action of statins and FTIs is likely to constitute a mechanistic basis for the synergistic effect with CHR2863, hence causing mTOR inhibition via amino acid depletion. Indeed, the present study showed [Figure 7] that the loss of Rheb prenylation provoked by simvastatin and FTI-277 was consistent with their potentiation effect on CHR2863 activity, whereas retention of Rheb prenylation by MVA or FFP abrogated this potentiating effect. However, it is remarkable that synergistic concentrations of simvastatin and CHR2863 had no apparent impact on Akt and mTOR phosphorylation patterns in U937 cells and CHR2863-resistant sublines. Therefore, further studies are required to identify other markers downstream of mTOR and to identify the mechanism of induction of apoptosis contributing to the synergy between statins and APis in AML cell lines and clinical specimens.

Many therapeutic interventions for AML are designed based on aberrant PI3K-Akt-mTOR signaling in AML cells[67]. Both statins and APis harbor properties interfering with this master regulator pathway and the current study provides a mechanistic rationale for their combination [Figure 8]. Whereas AML cells have shown heterogeneity in statin-induced apoptosis[29], the current study indicates that non-toxic concentrations of various statins synergize with APis in multiple AML cell lines. Non-toxic concentrations of statins, as employed in the *in vitro* studies, are readily achievable *in vivo*[26,68,69]. One earlier clinical study showed that Tosedostat combined with cytarabine or decitabine in untreated elderly AML or high-risk MDS patients was tolerated[14]; however, more recent studies revealed no survival benefit[16] or even inferior outcome in this patient category[18]. Although statin use was not reported in these studies, one could speculate that statin use, along with a high Tosedostat dosing, could contribute to over-potentiation of the drug. Therefore, it would be of interest to design a clinical study with lower doses of Tosedostat in a patient group of well-documented statin users to achieve an optimal potentiating effect and clinical benefit. Collectively, exploring the optimal combined efficacy of statins with APis in general and Tosedostat in particular deserves further exploration in the clinical setting of AML treatment.

In conclusion, this study revealed that non-toxic doses of statins could markedly potentiate the activity of aminopeptidase inhibitor (APi) drugs; both direct inhibitors like Bestatin and APi prodrugs like CHR2863 to (drug-resistant) human acute myeloid leukemia (AML) cells. The molecular basis underlying the potent synergistic inhibition of statins and APis on AML cells involved a dual inhibitory effect of impaired Rheb prenylation abrogating mTOR activation and APi-dependent mTOR inhibition. Given the fact that many cancer patients take statin medication for the treatment of other comorbidities, these novel findings call for awareness of the synergistic drug action of statins with APi-containing chemotherapeutic regimens and/or potential toxicities. These notions may warrant further evaluation in clinical studies including APis.

DECLARATION

Acknowledgments

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Availability of data and materials

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Conflict of interest

A preliminary account of this work was presented at the 2018 Annual Meeting of the American Society for Hematology (J. Cloos *et al*. Blood, vol 132, Supplement 1, Nov 2018, p 3945, abstract).

Ethical approval and consent to participate

This study only used cell lines, and thus ethical approval and consent to participate do not apply.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Harnessing the value of TCTP in breast cancer treatment resistance: an opportunity for personalized therapy

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Abstract

Early identification of breast cancer (BC) patients at a high risk of progression may aid in therapeutic and prognostic aims. This is especially true for metastatic disease, which is responsible for most cancer-related deaths. Growing evidence indicates that the translationally controlled tumor protein (TCTP) may be a clinically relevant marker for identifying poorly differentiated aggressive BC tumors. TCTP is an intriguing protein with pleiotropic functions, which is involved in multiple signaling pathways. TCTP may also be involved in stress response, cell growth and proliferation-related processes, underlying its potential role in the initiation of metastatic growth. Thus, TCTP marks specific cancer cell sub-populations with pronounced stress adaptation, stem-like and immune-evasive properties. Therefore, we have shown that *in vivo* phospho-TCTP levels correlate with the response of BC cells to anti-HER2 agents. In this review, we discuss the clinical relevance of TCTP for personalized therapy, specific TCTP-targeting strategies, and currently available therapeutic agents. We propose TCTP as an actionable

clinically relevant target that could potentially improve patient outcomes.

Keywords: Breast cancer, metastasis, TCTP, stem cells, therapy resistance, biomarker

INTRODUCTION

Identification of crucial genes with therapeutic implications at an early stage of disease is a central challenge in tailoring therapeutic strategies. Estrogen receptor (ER) expression, progesterone receptor (PR) expression, and human epidermal growth factor receptor-2 (HER2) overexpression or amplification are well-established biomarkers that drive treatment decisions for patients with breast cancers (BC)[1]. Multi-parameter genomic assays, such as Oncotype DX and MammaPrint, are being used for patients with hormone receptor-positive (ER^+/PgR^+) and $HER2^-$ early breast cancer who may benefit from adding chemotherapy to adjuvant endocrine therapy[2,3]. Specific gene signatures are still missing for non-ER^+/HER^- clinical BC subgroups because, among those reported in the literature, none are in clinical use[4].

Besides genomic studies, transcriptomic, proteomic and metabolomic analysis, or multi-omics data are all useful to discover biomarkers with an unprecedented level of complexity, as required for the current knowledge landscape of diseases. Further, post-translational modifications or subcellular localizations shape the level and the functional state of a protein, which cannot be detected by genomics-based approaches[5]. Last but not least, the relationship between protein and mRNA is very complex and highly influenced by cell types and cell states. Thus, transcript levels by themselves should not be deemed sufficient to predict protein levels[6,7].

There are two current frontiers of cancer therapy, on one side, identifying clinically relevant alteration and, on the other side, doing this as early as possible during the progression of the disease. For example, despite advances in breast cancer detection and treatment, predicting which patients will develop overt metastatic disease remains a challenge. This is not simply a formidable technological challenge; it implies fundamental questions related to the degree of similarity between metastatic tissues and its primary source. However, it appears clear that prometastatic mutations may be represented very early in the primary tumor. For instance, Estrogen Receptor 1 (ESR1) mutation status, which has been associated with acquired resistance to endocrine therapy, is present not only in metastatic lesions but also in primary BC[8].

After this general introduction, we intend to make the case here for translationally controlled tumor protein (TCTP), whose complex biology and link to clinical parameters may represent a good and practical example of the mentioned concepts.

We start from clinical observations highlighting the promising role of TCTP as a clinically relevant prognostic and predictive biomarker for identifying BC tumors at a high risk of progression early on. We also discuss signaling pathways that may impinge on stemness, drug resistance and poor response to immunotherapy. Finally, we explore strategies for targeting TCTP and the consequences of affecting TCTP expression in aggressive cancer cells.

CLINICAL RELEVANCE OF TCTP IN CANCER

TCTP, also known as histamine-releasing factor (HRF), fortilin, P23, is encoded by the *TPT1* gene located on chromosome 13q12→q14[9]. The gene produces two TCTP mRNA isoforms which are different in the length of their 3'-UTR [Figure 1]. In this present work, we will refer, for convenience, to the shorter isoform, which is the most generally expressed in normal and cancer tissues[10].

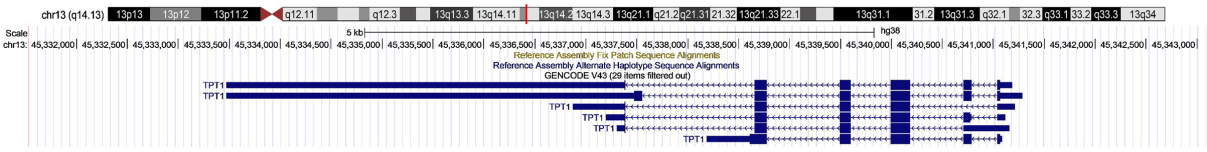

Figure 1. Genomic organization of TPT1. The UCSC genome browser was used to display the location and genomic organization of TPT1 on chromosome 13q14. Scheme of the TPT1 major types of alternative splicing is displayed (http://genome.ucsc.edu). UCSC Genome Browser on Human (GRCh38/hg38). TPT1: Tumor protein, translationally-controlled 1.

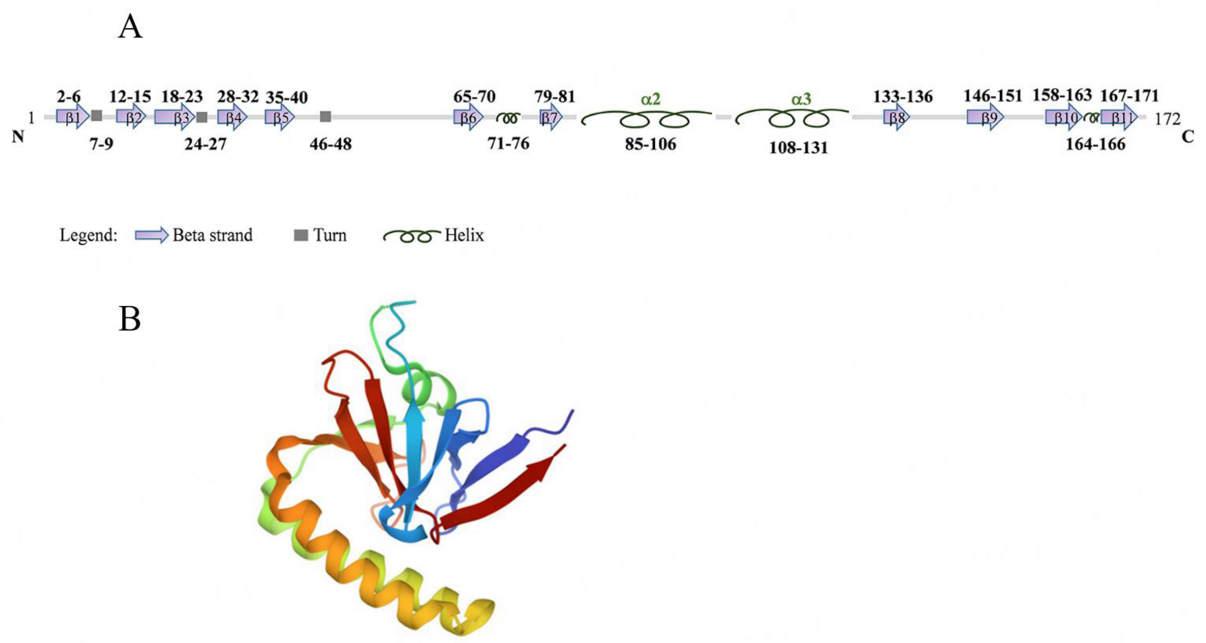

Figure 2. The peculiar structure of TCTP. (A) A cartoon representation of the secondary structure of human TCTP. Reference sequence from UniProt P13693[11]. β-strands, α-helices and turns elements are indicated in the legend. The N-terminal region includes a flexible loop extending from β5 to β6 strands. It contains a highly conserved signature and the Ser46 and Ser64 residues which are phosphorylated by the polo-like kinase Plk1, a crucial player in mitosis. A second conserved signature is found in the C-terminal region; (B) The crystal structure of the human TCTP (PDB Code 1YZ1[12]) discloses the α-helical hairpin (formed by Helix H2 and Helix H3), whose structure is similar to the H5-H6 helices of BCL-2 family proteins, and the β-stranded domain that shows a structural analogy with the guanine nucleotide exchange factors (GEF) Mss4/Dss4 protein families[13], suggesting a similar role for TCTP as GEF for Ras homolog enriched in brain (Rheb) in the mTORC1 pathway[14]. The flexible loop is not detectable in the crystal structure. TCTP: Translationally controlled tumor protein.

The gene encodes the protein TCTP, which is highly conserved throughout evolution and without any sequence homology to other proteins [Figure 2].

TCTP as a prognostic marker

Emerging clinical evidence has shown that the TCTP protein expression was dysregulated in many diseases[15], including various hematological[16,17] and solid tumors [Table 1]. Further, a high TCTP status was positively correlated with the pathological grade and markedly associated with shorter overall survival [Table 1]. Interestingly, the levels of TCTP were higher in metastatic lesions than in the corresponding primary gallbladder (GBC)[22] and colorectal carcinoma (CRC)[27].

Table 1. TCTP expression and clinical outcomes

Cancer	Expression level	Histological and prognostic features	Refs
Breast	High	Poor differentiation Short survival	[18] [19]
Epithelial ovarian cancer	High	Poor differentiation Short survival Lymph node metastasis	[20]
Cervical cancer	High	Poor differentiation Lymph node metastasis	[21]
Gallbladder cancer	High	Poor differentiation Metastasis Short survival	[22]
Pancreatic ductal adenocarcinoma	High	Lymph node metastasis Poor differentiation	[23]
Glioma	High	Poor differentiation Short survival	[24] [25]
Colorectal cancer	High	Poor differentiation Metastatic Short survival	[26] [27]
Prostate	High	Poor differentiation Metastasis Short survival	[28] [29]
Lung adenocarcinoma	High	Poor differentiation Short survival	[30]
NSCLC	High	Tumor size Short survival	[31]
Cholangiocarcinoma	High	Short survival	[32]
Neurofibromatosis type 1	High	Malignant phenotype	[33]
Neuroblastoma	High	Poor differentiation Short survival	[34]

TCTP: Translationally controlled tumor protein.

In breast cancer tissues, TCTP expression levels were higher compared to the corresponding normal tissues, and, notably, a high TCTP status was positively correlated with the pathological grade and was associated with shorter overall survival[18]. Interestingly, TCTP was enriched in PKH26 dye-retaining human normal mammary stem cells[35]. Further, TCTP enrichment was recorded in poorly differentiated high-grade BC tumors[18], the latter known to contain more cancer stem cells (CSCs) than lower-grade tumors[35]. Relevant to this, a high TCTP status has also been correlated to the presence of mutated P53 tumors and with high proliferative activities[18]. Loss of p53 function enabled acquisition of stem cell properties and led to Myc activation, thereby increasing the expression of a mitotic signature identifying BC patients at high risk of mortality and relapse[36].

Moreover, TCTP mRNA levels were significantly upregulated in ovarian cancer (OC) organoids generated from pluripotent stem cells (iPSC) of patients bearing the germline pathogenic breast cancer susceptibility gene 1 (*BRCA1*) mutation[37]. These findings highlight the potential role of TCTP as a biomarker in OC since patient-derived-organoids (PDOs) are a clinically valuable representation of the sourcing tumor[38]. This again echoes the above-mentioned data showing that TCTP levels are higher in poorly differentiated tissues compared to the well-differentiated ones in several tumors. Understanding the relationships between the BRCA1 genomic status and the higher level of TCTP in prognostic terms, in OC and additional tumors, may represent an important future investigation.

TCTP as a predictive marker

We have shown that TCTP, in the phosphorylated form, was a clinically relevant biomarker for a more aggressive BC[19]. TCTP was specifically phosphorylated by Polo-like kinase 1 (PLK1) on Ser46 and Ser64 residues[39-41], both located in the flexible loop of the protein, as reported by Malard *et al.*[42]. PLK1 was a crucial player in mitotic progression[43]. It was highly expressed in preinvasive in situ breast carcinomas[44], and correlated with high Ki-67 levels, TP53 mutations and poor clinical outcomes in primary BC[45,46]. Further, overexpression of PLK1 played a critical role in tumors that have escaped estrogen deprivation therapy[46,47], and was a strong predictor of worse survival in a large cohort of ER-positive BC patients[46-48].

We and others have shown that inhibition of PLK1 impaired TCTP phosphorylation[19,39,40], suggesting that a functional PLK1/TCTP axis could be critical for cancer progression. Consistently, both high levels of phospho-TCTP and PLK1 were found in neuroblastoma from patients with adverse prognostic factors[34], in agreement with our data showing that high levels of phospho-TCTP were correlated with high histological grade and with worse pathological parameters in primary BC tissues. Interestingly, the number of phospho-TCTP positive cells significantly increased (> 10%) when tumors were resistant to trastuzumab therapy (first line of treatment) and progressed towards the metastatic stage [Figure 3][19]. These findings suggest that phospho-TCTP may be a promising independent prognostic biomarker and a possible predictor of response to treatment. Tumors endowed with a higher fraction of cancer cells with a positive phospho-TCTP staining may exhibit increased drug resistance. A high phospho-TCTP status may allow classification of patients into responders and non-responders for trastuzumab therapy. Thus, targeting these cells with dihydroartemisinin (DHA) (see below) may improve long-term clinical outcomes, as recently suggested by our findings in HER2+BC cells resistant to trastuzumab therapy[49].

TCTP as a non-invasive diagnostic biomarker

Accessible biomarkers are a valuable and promising non-invasive tool for early diagnosis of BC. Serum tumor biomarkers such as carcinoembryonic antigen (CEA), cancer antigen (CA)-15-3, and CA-125 have been used for early detection of metastatic breast cancer (MBC). However, expression levels of these markers may be affected by anti-hypertensive medications or inflammatory diseases[50], thus raising concerns about their diagnostic accuracy.

TCTP has been shown to be secreted during allergic reactions and to promote immunoglobulin E (IgE)-mediated activation of mast cells and basophils. Its levels also increased in the serum of patients affected by rheumatoid arthritis (RA), suggesting a potential role as a biomarker in autoimmune and inflammatory disease[51,52].

Recently, a transcriptome profiling analysis has shown that TCTP mRNA levels increased in the saliva of BC patients compared to healthy ones[53]. In addition, ischemia and hypoxia induced TCTP secretion in blood samples of CRC patients, suggesting that circulating TCTP levels may play a role in CRC progression[27]. Moreover, the levels of TCTP were higher in the plasma of patients with cervical cancer[21] or squamous cell carcinomas (SCCs)[54] when compared to healthy subjects, suggesting its potential role as a novel non-invasive biomarker in several cancer types.

Extracellular vesicles (EVs) have also been proposed as reliable diagnostics biomarkers for the early detection of cancer. EVs are a heterogeneous population of vesicles that comprise three main classes: exosomes, shed microvesicles, and apoptotic bodies. EVs play a role in regulating intercellular communications by transferring biological information like proteins, lipids, and nucleic acids between cells[55].

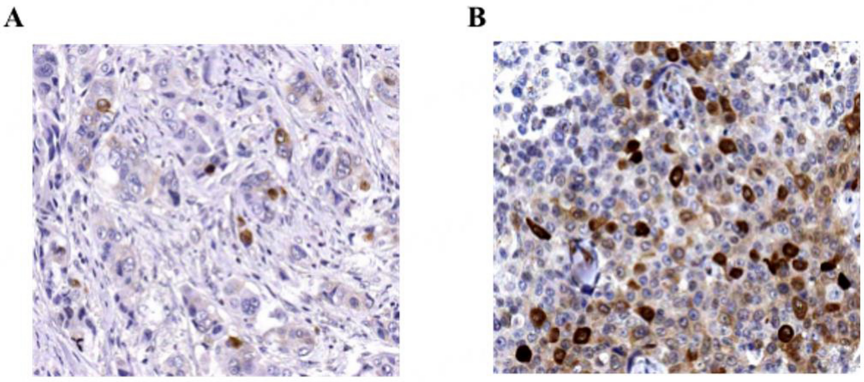

Figure 3. Correlation between Phospho-TCTP expression and response to trastuzumab therapy. Representative images of the immunohistochemical staining of phospho-TCTP in HER2 + BC patients (A) responsive and (B) non-responsive to trastuzumab. From Lucibello *et al.* "Phospho-TCTP as a therapeutic target of dihydroartemisinin for aggressive breast cancer cells". Oncotarget, 2015 [Figure 6][19]. BC: Breast cancers; HER2: human epidermal growth factor receptor-2; TCTP: translationally controlled tumor protein.

TCTP is a leaderless protein that could be secreted through the non-classical exosome pathway by cancer cell lines[56]. Recently, it has been shown that TPT1 transcripts were highly expressed and enriched in all EV subtypes from the human colon cancer LIM1863 cell line, thus speculating that it could be translated upon EV uptake in recipient cells[57]. It has been shown that the TCTP protein was sequestered into exosomes and released from endothelial cells in patients with pulmonary arterial hypertension (PAH). TCTP uptake from the circulation by pulmonary artery smooth muscle cells induced aberrant vascular remodeling, suggesting TCTP is a relevant biomarker in PAH disease[58].

Altogether, all these data suggest the potential of TCTP as a non-invasive biomarker in several diseases and pave the way for further studies strengthening its potential extracellular role in cancer diagnosis and prognosis.

TCTP as an immune-resistance factor

Oxidative stresses, lack of nutrients, radio and chemotherapy can trigger cell death responses. While programmed cell death ensures, at least initially, the removal of apoptotic bodies by phagocytes, death by necrosis results in the release of protein factors, called damage-associated molecular patterns (DAMPs), in the extracellular environment. These proteins have a well-defined intracellular function. However, when they are released in the tumor microenvironment (TME) through interaction with specific receptor molecules, such as Toll-like receptors (TLRs) or receptors for advanced glycation products (RAGE) present on epithelial cells or resident inflammatory cells, they induce recruitment of immune-inflammatory cells. Among them, there are immune cells with potent immunosuppressive activities as the myeloid-derived suppressor cells (MDSCs)[59,60].

Recently, it has been shown that TCTP was a crucial immunosuppressive danger factor released by dying tumor cells in the TME. TCTP promoted the recruitment of polymorphonuclear MDSCs (PMN-MDSC) into TME, which in turn blocked the antitumor function of CD8+T cells and NK cells. In detail, TCTP, through its binding to TLR2 on myeloid cells, stimulated the CXCL1 family chemokine expression, which in turn recruited PMN-MDSCs. This specific pathway was sufficient for the recruitment of PMN-MDSCs as no additional signals, such as G-CSF and GM-CSF, were required[61]. Notably, in this setting, the inhibition of TCTP, with a specific monoclonal antibody (55F3) or with DHA (see below), decreased MDSCs in the TME, inhibited tumor growth, thereby enhancing the efficacy of the immune checkpoint blockade (ICB)[61].

Thus, TCTP is a relevant player in such immunosuppressive circuits, and its inhibition may be effective in clinically viable combinatorial settings. Consistently, interrogating a TCGA colorectal data set showed that TCTP expression levels were negatively correlated with an antitumor immune signature of cytotoxic lymphocyte or NK cells. In CRC, a higher TCTP expression was elevated specifically in tumor cells and correlated with advanced disease and, notably, with PMN-MDSCs (CD15+ cells) representation within the tumor[61]. This latter observation correlates with the fact that the mesenchymal transition of high-grade breast carcinoma may be induced by MDSCs infiltrating the primary tumor[62].

The programmed death ligand-1 (PD-L1) is an immune checkpoint molecule and informative biomarker for anti-PD-1 therapy. Aberrant expression of PD-L1 may be induced through the dysregulation of several oncogenic pathways and contributes to immune escape[63,64]. TCTP was recently shown to induce resistance to anti-PD-L1 therapy, decrease T cell trafficking to the tumor and confer resistance to cytotoxic T lymphocyte-mediated tumor cell killing. In this frame, TCTP induced activation of EGFR-AKT signaling, and notably, the phosphorylation of TCTP by PLK1 was required for EGFR-AKT signaling and for establishing an immune-resistant phenotype[65]. It follows that inhibition of TCTP by DHA enhanced the efficacy of T cell-mediated therapy. Consistently, the expression of the TCTP mRNA was significantly higher in the patients unresponsive to anti-PD-L1 therapy, and it inversely correlated with T cell infiltration and CD8+T cell signatures in various types of cancer[65].

All these findings raise the intriguing possibility that TCTP might mark a subpopulation of cancer cells bearing immune-evasive properties. Finally, TCTP levels and status may predict the efficacy of anti-PD-1/PD-L1 immunotherapy, and, again, TCTP itself could be an actionable target for combinatorial therapies improving the response to T cell therapy or immune checkpoint blockade.

TCTP MAY MARK POORLY DIFFERENTIATED CELLS WITH STEM-LIKE PROPERTIES AND RESISTANCE TO THERAPY-INDUCED STRESS

Epithelial-mesenchymal transition (EMT) is a property of epithelial stem cells[66]. EMT is involved in tissue remodeling during embryonic development and in several pathophysiological processes in adult life, such as invasion and spread of tumor metastasis. During EMT, cells gradually acquire a mesenchymal cell phenotype characterized by a loss of contact with neighboring cells by changing cell shape and increasing the ability of migration and invasion. EMT is the first step towards metastasis. Disseminated cancer cells then undergo a mesenchymal-epithelial transition (MET) to re-initiate tumor growth. Epithelial and mesenchymal states are not endpoints of a transition but rather the expression of reversible phenotypic states. This phenotypic plasticity enables cancer cells to adapt to specific microenvironments, metabolic, immune, and therapeutic challenges[67-69]. Reactivation of developmental programs may be a critical step in the progression of cancers. Thus, genes and signaling pathways playing key roles in embryonic development pathways are often reactivated or dysregulated during tumorigenesis and metastasis[70].

TCTP knockout mice and TCTP-deficient mutants of Drosophila died in the early stage of embryogenesis[71,72], suggesting defects in embryo development. A recent study by Kwon YV and colleagues has shown that TCTP was required for maintaining Drosophila intestinal stem cells (ISCs) during normal homeostasis and tissue damage. In such a setting, TCTP increased Akt1 levels and its phosphorylation, which in turn promoted stem cell proliferation[73]. TCTP was enriched in PKH26 dye-retaining breast cancer stem cells, as previously mentioned[18], and in glioma stem cells[74].

TCTP has been shown to be a positive regulator of EMT. TCTP overexpression in polarized epithelial LLC-PK1 cells enhanced their cell motility, and invasiveness via mTORC2/Akt/GSK3b/b catenin pathway and promoted EMT-related markers and morphological changes[75]. TCTP was a target of transforming growth factor-β1 (TGF-β1), a potent inducer of EMT, in lung carcinoma cells. TCTP overexpression induced the protease urokinase plasminogen activator (uPA), which in turn induced extracellular matrix degradation, up-regulation of mesenchymal markers like vimentin, and reduction in epithelial markers, such as E-cadherin[76].

Several studies have shown a strong correlation between the expression levels of TCTP and the degree of metastasis [Table 1]. Importantly, silencing of TCTP suppressed pulmonary metastasis in melanoma-bearing mice[75], or liver metastasis in an *in vivo* model of GBC metastasis[22]. These data highlight the role of TCTP in the progression of the disease and its potential as a target in metastatic lesions.

Reorganization of cytoskeleton and changes in cell shape are essential for cancer cell mobility[67]. In Xenopus XL2 cells, TCTP was localized in a subset of actin-rich fibers of migrating cells, therefore, may regulate cell shape. Indeed, its reduction in XL2 and HeLa cells provoked drastic MT-dependent shape change[77]. Along this line, in LLC-PK1-renal proximal tubular epithelial cells, overexpression of TCTP induced a rearrangement of actin cytoskeleton and formation of stress fibers, which played a crucial role in cell mobility regulation[75].

We have observed that depletion of TCTP in breast cancer cells induced alterations in cell morphology, specifically an enlarged size and flattened shapes[78]. Notably, in several cancer cells, a decrease in TCTP levels significantly reduced cell migration and invasion[22,31,75]. We have also shown that a reduction of TCTP in its phosphorylated form induced an increase in microtubule density.

Thus, the phosphorylated and non-phosphorylated forms of TCTP must be kept in equilibrium to maintain the dynamic instability of microtubules and the cell shape. We also found that phospho-TCTP activity was crucial during mitosis, reminiscent of its mentioned link to cell proliferation[49].

We have also observed that TCTP is a critical survival factor that protects cancer cells from oxidative and metabolic stresses. Notably, arsenic trioxide (ATO), a pro-oxidant agent, induced up-regulation of TCTP, suggesting that chemotherapeutic treatments, through induction of TCTP expression, may select cells with a survival advantage[78]. This is intriguing since adaptation to stress and invasive ability are recognized features of stem-like cancer cell subpopulations. In this context, it is worth noting that EMT was shown to be an important propeller of cancer stem cell emergence[66].

Consistent with these data, in several tumors such as breast, lung, CRC and melanoma, a high TCTP status increased resistance to radio- and/or chemotherapy[79-82].

In chemo-resistant cells, the induction of TCTP was regulated through phosphoinositide-3-kinase (PI3K)/Akt/mTORC1 pathways[79], whose aberrant activation during malignant progression might result in loss of control of cell growth and survival, and the development of drug resistance[83].

Radiation may induce DNA damage by generating reactive oxygen species (ROS). TCTP knockdown sensitized cancer cells to radiation-induced DNA damage, reminiscent of its cytoprotective role under oxidative stress conditions[78], and activated p53, which in turn prevented proliferation of damaged cells[81], consistent with data showing that p53 and TCTP work as reciprocal regulators[18].

High levels of TCTP correlated with resistance to radiotherapy in high-grade glioma patients[25]. High-grade glioma tumors are characterized by an enrichment of CD133-positive cells with stemness properties, which may persist after therapy leading to tumor relapse[84]. In this context, TCTP could play a crucial role, as it has been shown that it is essential for cell proliferation and survival of primary glioma CD133-positive cells[74].

Altogether, these findings suggest that high TCTP levels could delineate a population of stem-like cancer cells with metastatic potential and pronounced stress adaptive properties, including chemo- and radio-resistance. This fits well with the above observations that TCTP could be a relevant indicator of high-grade malignancy.

Genomic analysis of TCTP in BC tumor

The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) or Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) databases showed a higher expression of TPT1/TCTP in normal breast tissues than in primary breast tumors. In addition, these analyses showed no differences in mRNA levels: (i) among the PAM50 subtypes of BC; (ii) among specific stages of the tumor; (iii) in metastatic *vs.* no metastatic tumors; (iv) in TP53 mutated tumors *vs.* TP53 wild-type tumors [Figure 4].

Moreover, the survival analysis from the Kaplan-Meier plot showed a positive correlation between high TPT1/TCTP mRNA levels and patient survival [Figure 5]. All these data are in apparent disagreement with the aforementioned data and suggest that mRNA levels of TCTP do not predict protein levels[6].

Things change when considering specific features of BC patients: when the analysis was restricted to estrogen receptor (ER)-negative tumors, characterized by more aggressive behavior [Figure 6], a high TCTP status positively correlated with shorter overall survival, thus suggesting the high degree of heterogeneity among BC subtypes.

In OC, instead, high levels of TCTP mRNA and protein were both significantly associated with poor overall survival [Figure 7]. Since OC is characterized by a history of sequential relapses after a second-line therapy[85], and given the involvement of OC cancer stem cells (CSC) in OC relapse[86], this indirectly links TCTP to the CSC biology, thus echoing the previous observation. See the availability of data and material for more details.

STRATEGIES TARGETING TCTP

For a more systematic view of the potential therapeutic approaches toward TCTP, we will divide the following discussion into subparagraphs.

Non-coding RNA

Non-coding RNA (ncRNAs) play key regulatory roles in oncogenesis through epigenetic, transcriptional, post-transcriptional, and translation modulation. ncRNAs can be used as biomarkers or can be therapeutically targeted in cancer therapy[87]. They are grouped into two main types according to transcript size: small (< 200 nucleotides; ncRNAs) and long (> 200 nucleotides; lncRNAs)[88].

Post-transcriptional gene regulation of TPT1 by potential ncRNAs in breast cancer remains largely unexplored. Recent works have shown that the lncRNA TPT1 antisense RNA 1 (TPT1-AS1) was dysregulated in tumors and associated with patient prognosis and clinically relevant features[89]. Conversely to TCTP, TPT1-AS1 was downregulated, and this predicted poor prognosis in BC. TPT1-AS1 played a role

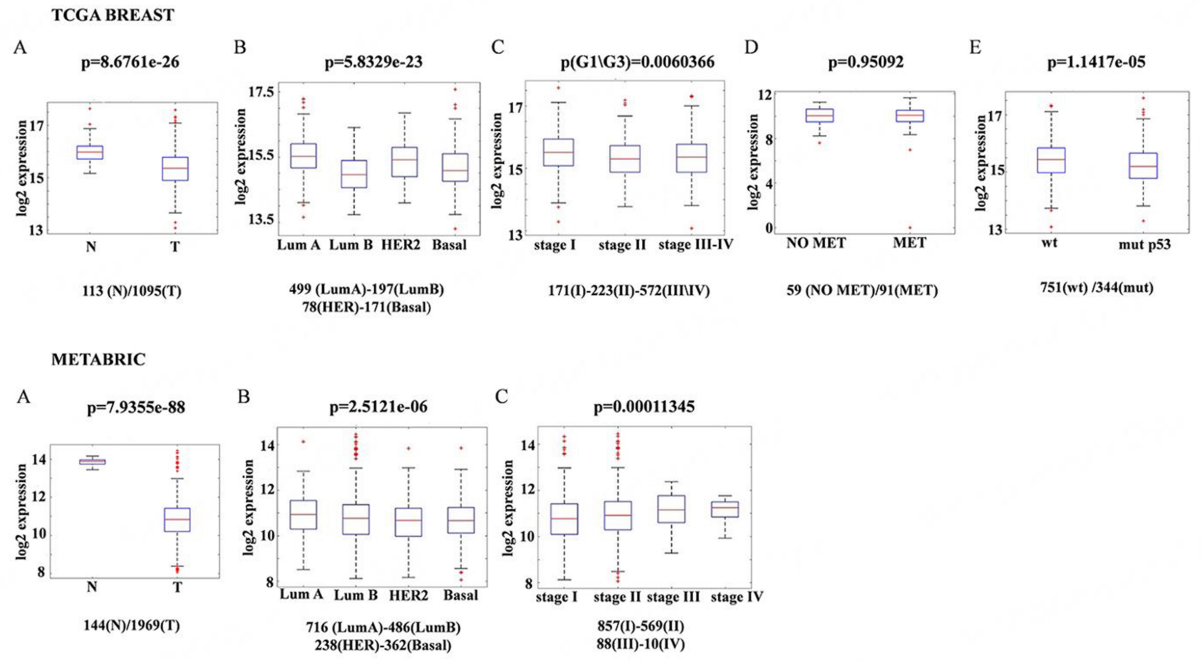

Figure 4. TPT1/TCTP transcripts in BC and normal tissues. Boxplot from TPT1/TCTP RNAseq gene expression data of TCGA (upper panel) and METABRIC databases (Lower panel). (A) in normal tissue and primary breast cancer tissue; (B) in different PAM50 breast cancer subtypes; (C) in different stages of the tumor; (D) in metastatic (91 samples) *vs.* no metastatic tumors (59 samples); (E) in TP53 mutated tumors *vs.* TP53 wild-type tumors. BC: Breast cancers; MET: mesenchymal-epithelial transition; METABRIC: molecular taxonomy of breast cancer international consortium; TCGA: the cancer genome atlas; TCTP: translationally controlled tumor protein; TPT1: tumor protein, translationally-controlled 1.

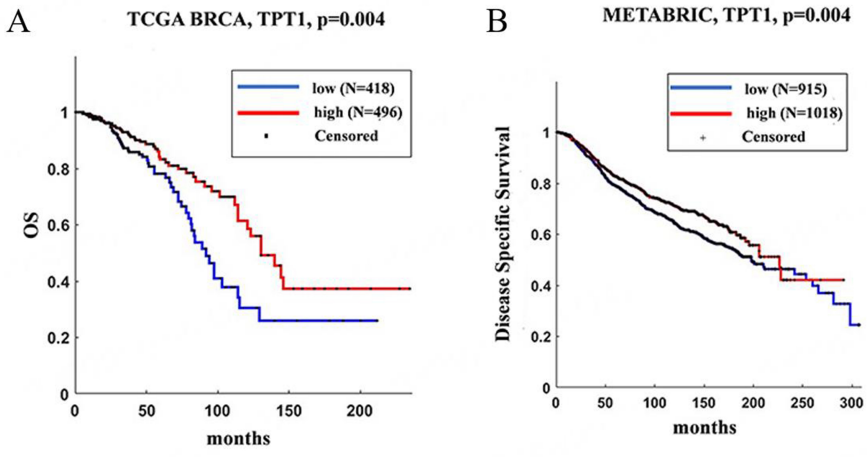

Figure 5. High TCTP levels correlate with better clinical outcomes in BC patients. (A) TCGA survival curve (B) and Disease-Specific Survival in METABRIC dataset of BC patients separated according to TPT1 expression levels. BC: Breast cancers; BRCA: breast cancer susceptibility gene; METABRIC: molecular taxonomy of breast cancer international consortium; TCGA: the cancer genome atlas; TCTP: translationally controlled tumor protein; TPT1: tumor protein, translationally-controlled 1.

as a tumor suppressor gene[90,91], and notably, it was subjected to epigenetic regulation through DNA methylation. Elango and colleagues have shown that TPT1-AS1 was the most induced lncRNA in response to DNA methyltransferase inhibition in triple-negative breast cancer (TNBC) models[92]. DNA methyl transferase inhibitors reverse epigenetic alterations, resulting in reactivation of tumor suppressor genes,

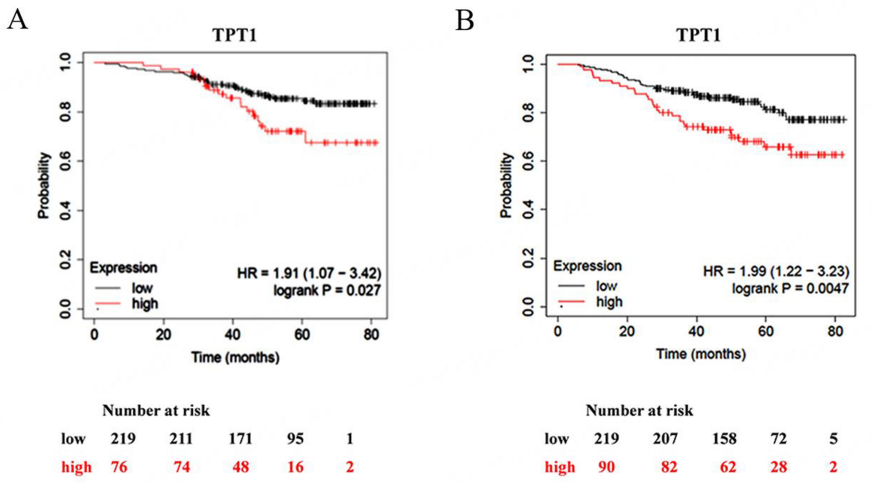

Figure 6. Overall survival is shorter in BC patients with higher TCTP expression. Kaplan-Meier analysis of overall survival in BC patients based on KM plotter database of: (A) PAM50 HER2 and (B) PAM50 basal subtypes. BC: Breast cancers; HER2: human epidermal growth factor receptor-2; TCTP: translationally controlled tumor protein; TPT1: tumor protein, translationally-controlled 1.

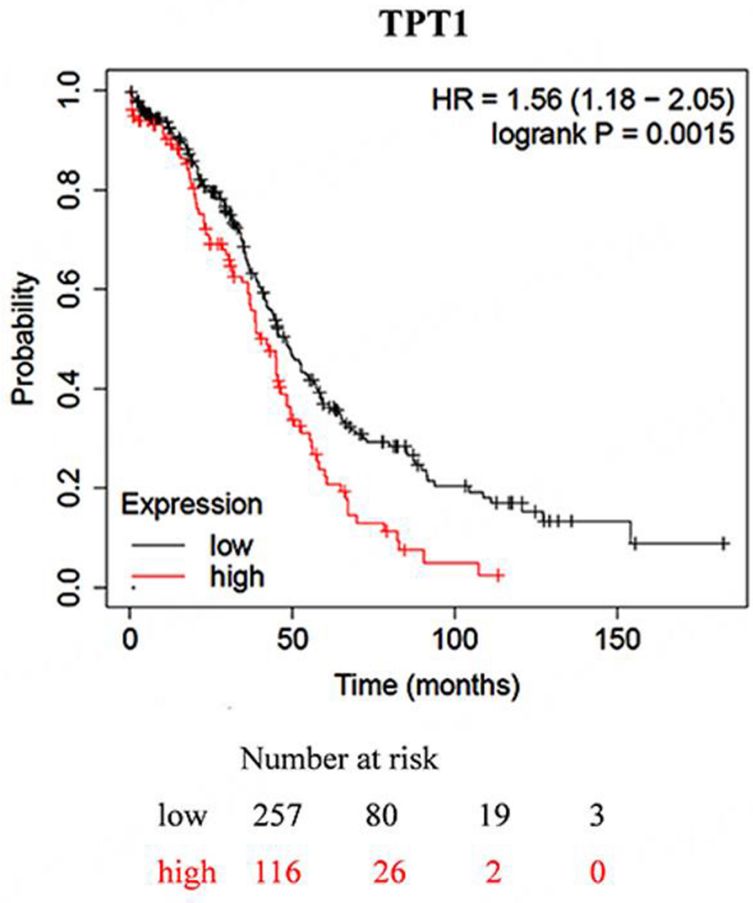

Figure 7. Overall survival is shorter in OC patients with higher TCTP expression. Kaplan-Meier analysis of overall survival in OC patients based on KM plotter dataset. OC: Ovarian cancer; TCTP: translationally controlled tumor protein; TPT1: tumor protein, translationally-controlled 1.

which in turn leads to cell cycle arrest and/or apoptosis, and thus suggests that re-expression of TPT1-AS1 could be an approach to be pursued in breast cancer therapy.

More in detail, the TPT1-AS1 is a transcript from the antisense strand of *TPT1* gene that may positively regulate the expression of TPT1. Consistent with this, TPT1-AS1 promoted tumor progression by upregulating TPT1 levels in epithelial ovarian cancer (EOC). In addition, the high expression of TPT1-AS1 was associated with unfavorable clinic-pathological features and poor prognosis in EOC[93]. The dual functions of TPT1-AS1 suggest that its role should be carefully considered in a tissue-specific manner. Further studies are therefore required to characterize the complex interaction network that regulates TPT1-AS1 activity in a specific tissue or context and to understand its underlying mechanisms.

Clinically available compounds

Drug repositioning is a promising strategy to identify new therapeutic applications of drugs already approved by regulatory agencies for other diseases. The use of clinically approved or advanced phase compounds is justified by the need to maximize the timing for therapeutic advancements since those compounds already have a consolidated safety profile and a characterized pharmacokinetic/pharmacodynamic profile. Thus, those compounds are in principle amenable to phase 2 studies. Below we will list some of the compounds falling into this category, whose mechanism of action is directly or indirectly linked to perturbing the levels or post-translational status of TCTP [Tables 2-4].

Dihydroartemisinin

One clinically available TCTP-targeting agent is DHA [Figure 8], the active metabolite of all artemisinin compounds (as artesunate, artemether). Artemisinin is the active principle in Artemisia annua. Artemisinin and its derivatives are a family of sesquiterpene trioxane lactone. These agents were discovered as anti-malarial agents by Dr. Youyou Tu, who received the Nobel Prize in Physiology or Medicine in 2015. Currently, the water-soluble derivative of artemisinin, Artesunate Amivas, has received approval for malarial patients from the Food and Drug Administration (FDA) and the European Medicine Agency (EMA).

Beyond the "anti-malarial effect", these pharmacological compounds were selectively cytotoxic to cancer cells, as shown by numerous *in vitro* and *in vivo* studies[94,95], which have led to the groundwork for the design of phase 1 clinical trials for solid tumors. These compounds have exhibited a good therapeutic index after long-term treatment in patients with metastatic breast cancer or cervix carcinoma[96-98].

Several observations have shown that DHA is bound specifically to TCTP[99-101]. Further, DHA inhibited TCTP-dependent cell migration and invasion of GBC cells, and notably, DHA decreased GBC metastases and improved survival in tumor-bearing mice. TCTP is a clinically relevant target in GBC[22]; thereby, TCTP-targeting DHA could be an anti-metastatic strategy to be explored at an early stage of disease.

We have shown that the levels of phospho-TCTP were crucial for the mitotic process. By decreasing phospho-TCTP levels, DHA induced mitotic spindle aberrations and the formation of disorganized microtubule structures[49]. At least partially through this mechanism, DHA increased the sensitivity of BC cells to chemotherapy[19] and to trastuzumab emtansine (T-DM1) in BC cells resistant to trastuzumab (first-line of treatment)[49]. T-DM1 is an anti-human epidermal growth factor receptor 2 (HER2) antibody-drug with a stable linker to emtansine (DM1), a microtubule inhibitor. Recently, it has been shown that T-DM1 reduced the risk of disease recurrence in patients with residual invasive BC after neoadjuvant therapy (NAT) comprised of HER2-targeted therapy and chemotherapy[102]. As partially suggested before, we may

Table 2. DHA as a repurposed drug targeting TCTP

Effect	Studies
Inhibition BC cell growth. Chemosensitization	Lucibello *et al.* 2015[19]
Inhibition of GBC migration and invasion. Inhibition of GBC metastasis. Improved survival in mice	Zhang *et al.* 2017[22]
Enhancement of anti- HER2 antibody therapies	D'Amico *et al.* 2020[49]
Inhibition of tumor growth Reduction of MDSCs in TME	Hangai *et al.* 2021[61]
Enhancement of the efficacy of T cell therapy	Lee *et al.* 2022[65]

BC: Breast cancers; DHA: dihydroartemisinin; GBC: gallbladder; HER2: human epidermal growth factor receptor-2; MDSCs: myeloid-derived suppressor cells; TCTP: translationally controlled tumor protein; TME: tumor microenvironment.

Table 3. Sertraline and thioridazine as repurposed drugs targeting TCTP

Effect	Studies
Inhibition cell growth and induction of apoptosis. Reduction of mammosphere-forming efficiency	Amson *et al.* 2012[18] Tuynder *et al.* 2004[105]
Inhibition of migration and invasion Inhibition of tumor growth	Boia-Ferreira *et al.* 2017[106]
Reduction of the number of prostate cancer stem cells	Chinnapaka *et al.* 2020[107]
Enhancement of the efficacy of DNA-damaging therapy and PARP1 inhibitor	Li *et al.* 2017[108]

TCTP: Translationally controlled tumor protein.

Table 4. Rapamycin as a repurposed drug targeting TCTP

Effect	Studies
Inhibition of MPNSTs cell growth *in vivo*	Kobayashi *et al.* 2014[33]
Enhancement of the efficacy of cisplatin and doxorubicin	Bommer *et al.* 2017[79]
Inhibition of cell growth	Bommer *et al.* 2015[113]

MPNSTs: Malignant peripheral nerve sheath tumors; TCTP: translationally controlled tumor protein.

speculate that the addition of DHA to T-DM1 may allow a dose reduction for T-DM1 and could help prevent adverse side effects, therefore improving the quality of life for patients.

Targeting TCTP with DHA may offer an unforeseen rationale for combination treatments in cancer immunotherapy as well, according to what was previously mentioned[61,65]. Immunotherapy represents a promising therapeutic approach for TNBC, and recently, pembrolizumab (anti-PD1 antibody) plus chemotherapy was approved for the treatment of advanced TNBC with high PD-L1 expression[103]. However, resistance to immune-checkpoint inhibitors (ICIs), including aberrant activation of oncogenic signaling pathways and/or an immunosuppressive TME, is a significant challenge. Predictive factors, as well as discovering new actionable targets for combinatorial therapy, will improve the response rate of TNBC to ICIs. In this context, TCTP appears to be an interesting subject of investigation. Thus, targeting TCTP may enable chemosensitization of cancer cells previously subjected to conventional therapy and to biological agents [Table 2].

Sertraline and thioridazine

Sertraline (Zoloft) is a selective serotonin reuptake inhibitor. Thioridazine is an antipsychotic drug used to treat schizophrenia. These agents, used in the management of psychiatric disorders, have shown promising anticancer effects as well[18,104].

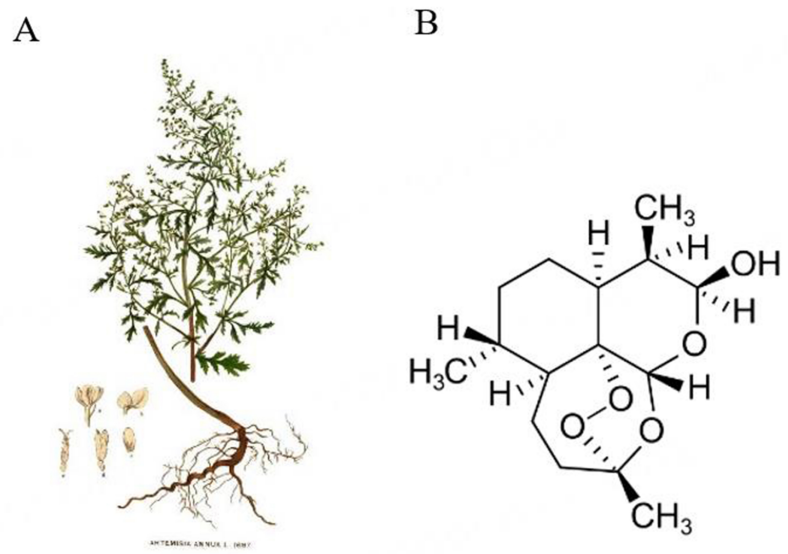

Figure 8. (A) Representative image of *Artemisia Annua*.https://commons.wikimedia.org/wiki/File:Artemisia_annua_-_001x.jpg. Created by: Oceancetaceen - Alice Chodura, Public domain, via Wikimedia; (B) Chemical structures of dihydroartemisinin. https://commons.wikimedia.org/wiki/File:Artenimol_(2).svg. Created by: Benff, CC BY-SA 4.0 <https://creativecommons.org.

Thioridazine and sertraline induced a significant reduction of TCTP levels, which in turn increased p53 levels, thus restoring the sensitivity of tumor cells to apoptosis. In addition, sertraline reduced the mammosphere-forming efficiency of ErbB2 cells, consistent with data showing that silencing of TCTP in ErbB2 cells resulted in a decreased ability to form mammospheres[18,105]. TCTP was also a critical target of sertraline and thioridazine in melanoma cells. Both drugs restored p53 function and inhibited invasion/migration and clonogenicity of melanoma cells by targeting TCTP. Notably, in vivo experiments on a mouse melanoma model have shown that sertraline was more effective in inhibiting tumor growth than dacarbazine, a chemotherapy agent that has been approved for treating advanced melanoma[106]. In prostate cancer cells, sertraline decreased TCTP, phospho-TCTP and survivin and induced apoptotic cell death. Further, sertraline inhibited the expression of cancer stem cell markers, CD44 and aldehyde dehydrogenase 1 (ALDH1), in prostate cancer cell cultures[107]. By using affinity purification-based proteomic profiling, Li *et al.* found that TCTP interacted with proteins involved in DNA repair. In breast cancer cells, inactivation of TCTP by sertraline enhanced UVC irradiation-induced apoptosis and increased the sensibility to etoposide and olaparib, a DNA-damaging drug and a PARP1 inhibitor, respectively[108]. Interestingly, such action may converge into the evoked chemosensitizing effect of TCTP [Table 3].

Whether sertraline and thioridazine may directly or indirectly perturb the function of TCTP is a matter of interesting ongoing debate[109].

Rapamycin

Rapamycin is a natural anti-fungal antibiotic. Rapamycin and its analogs are currently being used in clinical as potent immunosuppressants and antiproliferative agents. Rapamycin inhibits the Target of Rapamycin Complex 1 (TORC1) activity with high specificity. TORC1 is a highly conserved protein kinase that belongs to the PI3K family. It is a crucial regulator of cell proliferation and growth that integrates and senses different signaling networks. Its signaling dysregulation is frequently observed in several pathological conditions and in aggressive and therapy-refractory cancers, among these BC[83,110].

Studies in Drosophila melanogaster and structural studies in human models have shown the involvement of TCTP in TORC1 signaling. It has been reported that TCTP controlled cell growth and proliferation by positively regulating the Ras homolog enriched in brain (Rheb) activity, an important upstream activator of mTORC1[14,72,111,112].

In addition, TCTP mRNA belongs to the class of 5'-TOP mRNAs (containing a 5'-terminal oligopyrimidine tract, 5'-TOP) whose translational activity is largely controlled through the PI3-K/Akt/mTORC1 pathway[113]. In colon cancer cells and in HeLa cells, serum stimulation increased the expression of TCTP, and this was inhibited by rapamycin or mTOR kinase inhibitors. In addition, in colon cancer, TCTP protein levels were upregulated by the mTORC1 pathway in response to 5-fluorouracile (5-FU) and oxaliplatin treatment, suggesting a protective role of TCTP against the cytotoxic action of those anticancer drugs. Consistent with this data, mTOR kinase inhibitors prevented the onset of this TCTP-driven drug resistance phenotype[79].

Further, a positive feedback loop between TCTP and mTOR contributed to neurofibromatosis type 1 (NF1)-associated tumor, and rapamycin was effective in down-regulating TCTP expression, suggesting that the TCTP protein level was controlled by mTOR-dependent translational regulation[33].

Recently, it has been shown that Rapamycin induced TCTP proteolysis, thereby enhancing the efficacy of DNA-damaging drugs, such as cisplatin and doxorubicin, in lung carcinoma cells, suggesting a novel strategy for enhancing chemosensitivity in lung cancers[80].

Unfortunately, the mentioned compelling evidence has not yet generated clinically meaningful results, and this general observation is related to the poor activity of mTOR inhibitors observed so far in clinical trials. However, everolimus, an mTOR inhibitor, has been approved in combination with exemestane, an aromatase inhibitor, in $HR^+/HER2^-$ endocrine-resistant MBC[114].

It is possible that multiple TCTP targeting agents may be combined to increase the effect: for example, preclinical studies have shown that the combination of artesunate, a specific inhibitor of TCTP (see above), and rapamycin was more effective in killing malignant peripheral nerve sheath tumors (MPNSTs) compared to the effect induced by exposure to a single drug[33] [Table 4].

CONCLUSION

TCTP may mark specific subpopulations of cancer cells with pronounced stress adaptation, stem-like and immune-evasive properties and capable of surviving conventional agents or targeted therapies [Figure 9].

It may work by interacting with key factors involved in cancer progression, including metabolic modulators. TCTP may also be involved in the tumor-TME crosstalk [Figure 10], and it may aid in stratifying specific cancer subsets. Thus, further studying TCTP and its complex behavior in cancer may reveal important knowledge towards patient stratification for both therapeutic and prognostic purposes. We also evoke the need to study the complex biology and the TCTP-modulating agents in more clinically relevant models, such as patient-derived-organoids. This may aid in solving some of the apparent contradictions in the behavior of this interesting and complex protein, down to a tumor- and patient-specific level. This effort is currently ongoing in our labs. Altogether, we suggest that TCTP may mark an aggressive sub-population of cancer cells that could emerge under the pressure of conventional therapies. Additionally, based on the preclinical evidence, we propose that TCTP could be an actionable target of clinically available compounds aimed at targeting TCTP highly expressing tumors, with the purpose of attenuating their resistance to

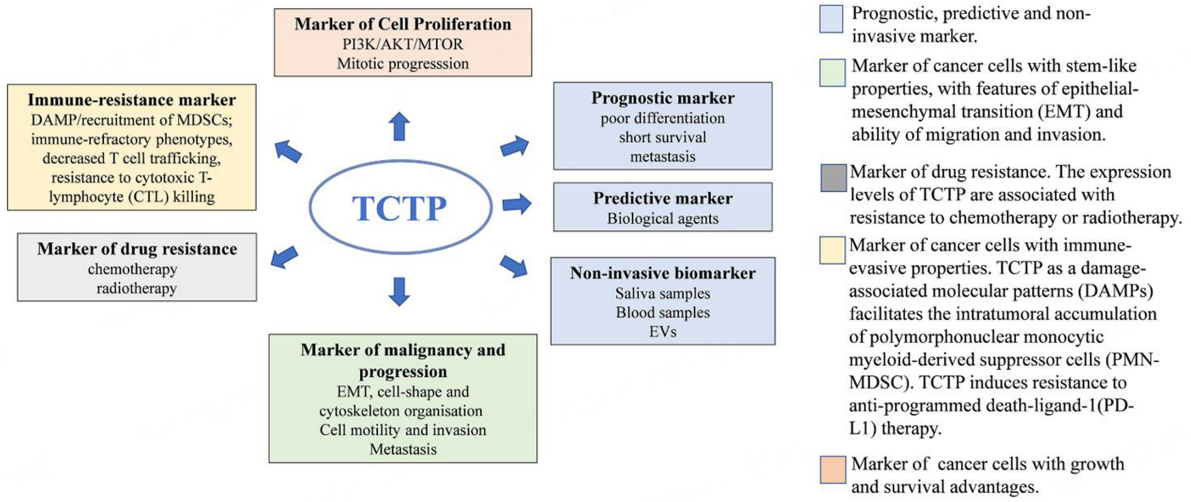

Figure 9. Schematic overview of the potential application of TCTP in cancer. EVs: Extracellular vehicles; MDSCs: myeloid-derived suppressor cells; TCTP: translationally controlled tumor protein.

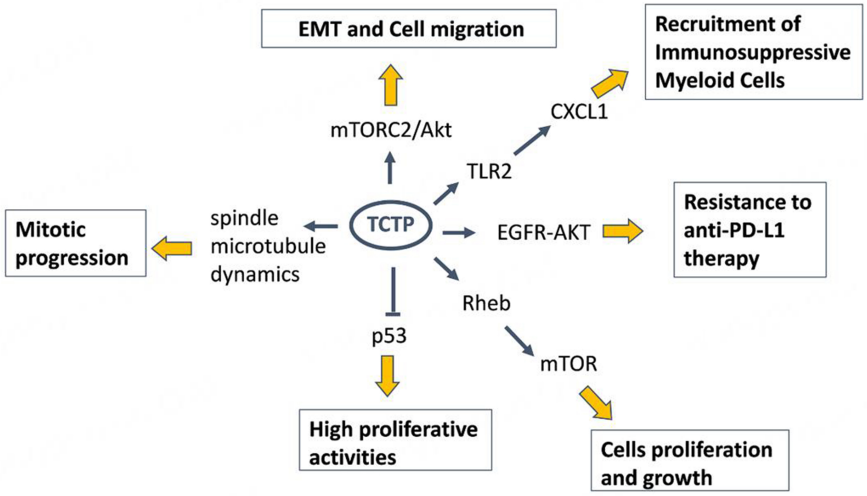

Figure 10. Pleiotropic functions of TCTP. A cartoon showing the major TCTP-related pathways. TCTP can promote cell proliferation by acting: (1) as a guanidine exchange factor for the GTP-binding protein Rheb, a crucial player in the mTORC1 pathway; (2) by inducing P53 degradation; (3) by regulating spindle morphology and mitosis progression, when it is phosphorylated by PLK1. TCTP can act as an immune-resistance factor via activation of TLR2 on myeloid cells, which in turn induces cytokines production and the recruitment of immune-suppressive cells. TCTP, in the phosphorylated form, can induce the activation of the EGFR/AKT pathway, thus promoting an immune resistance to anti-PD-L1 therapy. TCTP can promote cell migration and EMT markers through the mTORC2 pathways. EMT: Epithelial-mesenchymal transition; PD-L1: programmed death ligand-1; PLK1: polo-like kinase 1; TCTP: translationally controlled tumor protein.

therapy and progression. Targeting TCTP with DHA, sertraline or rapamycin holds promise for more effective synergistic combinations. This warrants further investigations, which are ongoing in our lab. Thus, we believe that two important factors may converge in delineating the study, and the targeting of TCTP can be an opportunity for personalized therapy. On one hand, the fact that TCTP marks a cell subpopulation of cancer cells, which represent obvious candidates for resistance to therapy, as also suggested by the prognostic value of both TCTP and phospho-TCTP levels; on the other hand, the availability of compounds with an established safety profile capable of reducing the levels of the protein and altering its

phosphorylation state. What is mentioned here may represent a starting point for considering TCTP measurement and targeting as a translationally relevant opportunity.

Statistics

Statistical analyses were performed using R software[115]. ANOVA or Student's *t*-test was used for normally distributed data and for non-normally distributed data, as appropriate. *P*-values smaller than 0.05 were considered statistically significant.

DECLARATIONS

Authors' contributions

Critical review, editing, commentary, and bioinformatic analysis: Santamaria G, Cioce M
Critical review, editing, and commentary: Rizzuto A, Fazio VM, Viglietto G
Conceptualization, literature search, original draft preparation, visualization, and editing: Lucibello M

Availability of data and material

Data from the METABRIC study is deposited in the European Genome-phenome Archive and can be downloaded from European Genome-phenome Archive: METABRIC, (https://www.ebi.ac.uk/ega/studies/EGAS00000000083). The TCGA breast cancer dataset can be downloaded from the TCGA Data Portal website, (http://tcga-data.nci.nih.gov/tcga/).

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Original Article

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The multidrug resistance transporter P-glycoprotein confers resistance to ferroptosis inducers

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Abstract

Aim: Ferroptosis is a non-apoptotic form of cell death caused by lethal lipid peroxidation. Several small molecule ferroptosis inducers (FINs) have been reported, yet little information is available regarding their interaction with the ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp, ABCB1) and ABCG2. We thus sought to characterize the interactions of FINs with P-gp and ABCG2, which may provide information regarding oral bioavailability and brain penetration and predict drug-drug interactions.

Methods: Cytotoxicity assays with ferroptosis-sensitive A673 cells transfected to express P-gp or ABCG2 were used to determine the ability of the transporters to confer resistance to FINs; confirmatory studies were performed in OVCAR8 and NCI/ADR-RES cells. The ability of FINs to inhibit P-gp or ABCG2 was determined using the fluorescent substrates rhodamine 123 or purpuin-18, respectively.

Results: P-gp overexpression conferred resistance to FIN56 and the erastin derivatives imidazole ketone erastin and piperazine erastin. P-gp-mediated resistance to imidazole ketone erastin and piperazine erastin was also reversed in UO-31 renal cancer cells by CRISPR-mediated knockout of *ABCB1*. The FINs ML-162, GPX inhibitor 26a, and PACMA31 at 10 μM were able to increase intracellular rhodamine 123 fluorescence over 10-fold in

P-gp-expressing MDR-19 cells. GPX inhibitor 26a was able to increase intracellular purpurin-18 fluorescence over 4-fold in ABCG2-expressing R-5 cells.

Conclusion: Expression of P-gp may reduce the efficacy of these FINs in cancers that express the transporter and may prevent access to sanctuary sites such as the brain. The ability of some FINs to inhibit P-gp and ABCG2 suggests potential drug-drug interactions.

Keywords: Ferroptosis, drug resistance, P-glycoprotein, ABCG2

INTRODUCTION

Ferroptosis is an iron-dependent form of non-apoptotic cell death arising from direct or indirect inhibition of glutathione peroxidase 4 (GPX4), leading to lipid peroxidation and unsustainable levels of reactive oxygen species (ROS)[1]. One of the first reported inducers of ferroptosis, erastin, was described before the concept of ferroptosis was completely understood. Erastin was found to selectively kill transformed human foreskin fibroblasts expressing mutant HRAS compared to isogenic cells expressing wild-type HRAS[2]. The mechanism of cell death induced by erastin was not apoptosis, as the hallmarks of apoptotic cell death such as annexin V staining and caspase 3 cleavage were not observed, although death was accompanied by cell membrane permeabilization[2]. The term "ferroptosis" was later coined for this novel form of cell death as iron chelators and antioxidants were found to potentiate erastin-mediated toxicity, suggesting an iron-dependent increase in ROS was responsible[3]. Additionally, ferroptosis could not be inhibited by caspase inhibitors and was found to occur independently of the apoptosis effector proteins Bak and Bax[3].

The target of erastin was identified to be the cystine/glutamate antiporter, system x^-_c[3]. Inhibition of the antiporter leads to depletion of glutathione and inactivation of GPX4[4]. Subsequent to the discovery of erastin, other small molecules have been developed to induce ferroptosis by direct or indirect inhibition of GPX4. These ferroptosis inducers (FINs) include modified forms of erastin such as erastin2[5], imidazole ketone erastin[6] and piperazine erastin[4], as well as the inhibitors FIN56[7], RSL3[8], $FINO_2$[9], PACMA31[10], GPX4 inhibitor 26a[11], and ML-162 and ML-210[12].

In an attempt to identify cancers that might be effectively treated by ferroptosis induction, Yang *et al.* tested erastin toxicity in 117 cancer cell lines and identified renal cell carcinomas as being particularly sensitive to ferroptosis[4]. This intrigued us, as renal cell carcinomas are often positive for expression of P-glycoprotein (P-gp, encoded by the *ABCB1* gene), an ATP-binding cassette (ABC) multidrug efflux pump that confers drug resistance[13,14]. Some reports have also suggested that expression levels of another ABC transporter, ABCG2 (encoded by the *ABCG2* gene), also expressed in kidney cancers, can predict overall survival in patients with clear cell renal carcinoma[15]. Additionally, both P-gp and ABCG2 localize to the gastrointestinal tract as well as to other barrier sites, such as the blood-brain barrier (BBB)[16]. Expression at these sites is linked to their role in limiting the oral bioavailability of chemotherapy drugs and brain penetration of several targeted therapies[16,17]. We thus sought to characterize the interactions between small-molecule ferroptosis inducers and the transporters P-gp and ABCG2.

METHODS

Chemicals

Erastin, doxorubicin, rhodamine 123, and FIN56 were purchased from Sigma-Aldrich (St. Louis, MO). Erastin2, imidazole ketone erastin, RSL3, ML-162, $FINO_2$, GPX4 inhibitor 26a, JKE-1674, and JKE-1716 were from Cayman Chemical (Ann Arbor, MI). Valspodar was obtained from MedChemExpress (Monmouth Junction, NJ). Romidepsin was from Selleck Chemicals (Houston, TX). Purpurin-18 was

purchased from Frontier Scientific (Logan, UT). Piperazine erastin was from TargetMol (Wellesly Hills, MA). SN-38 was obtained from LKT Laboratories (St. Paul, MN). RSL3 was purchased from Tocris (Minneapolis, MN). Fumitremorgin C (FTC, > 95% purity) was synthesized in-house by the Developmental Therapeutics Program at the National Institutes of Health (Bethesda, MD).

Cell lines

OVCAR8, NCI/ADR-RES and UO-31 cells were obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute (Frederick, MD) and are grown in RPMI-1640 with 10% FBS, glutamine and Pen/Strep. A673 cells (from ATCC, Manassas, VA) were seeded and transfected with empty vector (EV) or vector containing full-length, human *ABCB1* or *ABCG2* using Lipofectamine 2000 (Invitrogen, Waltham, MA). Cells were selected with hygromycin, and clones were isolated by limiting dilution. Selected clones were grown in DMEM with 10% fetal calf serum, glutamine and Pen/Strep, as well as 300 μg/mL hygromycin to maintain expression of the transporters. P-gp-overexpressing MDR-19 cells and ABCG2-overexpressing R-5 cells were derived from HEK293 cells and have been previously characterized and described[18]. Transfected HEK293 cells were grown in MEM with 10% fetal calf serum, glutamine and Pen/Strep along with 2 mg/mL G418 to maintain expression of the transporters. All cell lines were routinely tested for mycoplasma using the MycoAlert PLUS Kit (Promega, Madison, WI) test kit and their identities were confirmed by STR analysis (performed by ATCC, Manassas, VA).

Oligonucleotides

The following oligonucleotides (generated by Eurofins, Inc, Louisville, KY) were used in this study:

ABCB1-START:
5'- GGGGACAACTTTGTACAAAAAAGTTGGCACCATGGATCTTGAAGGGGACCGCAATGG

ABCB1-END:
5'- GGGGACAACTTTGTACAAGAAAGTTGATTATGCTAGCTGGCGCTTTGTTCCAGCCTGG

ABCG2-START:
5'- GGGGACAACTTTGTACAAAAAAGTTGGCACCATGTCTTCCAGTAATGTCGAAGTTTTTATCCC

ABCG2-END:
5'- GGGGACAACTTTGTACAAGAAAGTTGATTAAGAATACTTTTTAAGAAATAACAATTTCAG

Generation of entry clones

Entry clones for *ABCB1* and *ABCG2* were constructed by PCR amplification of cDNA sequences flanked by Gateway Multisite recombination sites (Thermo Fisher Scientific, Waltham, MA). PCR was carried out with 200 nM of each oligo listed in the table above using Phusion polymerase (New England Biolabs, Ipswich, MA) under standard conditions and an extension time of 180 s. PCR products were cleaned using the QiaQuick PCR purification kit (Qiagen, Germantown, MD). The final PCR products were recombined into Gateway Donor vector pDonr-253 using the Gateway BP recombination reaction using the manufacturer's protocols. The subsequent Entry clones were sequence verified throughout the entire cloned region.

Subcloning for mammalian expression constructs

Gateway Multisite LR recombination was used to construct the final mammalian expression constructs from the Entry clones using the manufacturer's protocols (Thermo Fisher Scientific, Waltham, MA). The Gateway Destination vector used was pDest-305 (Addgene. Watertown, MA, #161895), a mammalian

expression vector containing a Gateway attR4-attR2 cassette based on a modified version of pcDNA3.1. This vector backbone contains a hygromycin resistance marker for antibiotic selection. A human elongation factor 1 (EF1) promoter was introduced using a Gateway att4-att1 Entry clone (Addgene, Watertown, MA, #162920). Final expression clones were verified by restriction analysis and maxiprep DNA was prepared using the Qiaprep Maxiprep kit (Qiagen, Germantown, MD).

Generation of ABCB1 knockout UO-31 cells

CRISPR-mediated knockout of *ABCB1* in UO-31 cells was achieved by co-transfecting cells with knockout and homology-directed repair vectors for *ABCB1* (obtained from Santa Cruz Biotechnology, Dallas, TX) using Lipofectamine 2000 (Invitrogen) and subsequent selection with puromycin (3 μg/mL). Knockout clones were subsequently isolated, and loss of P-gp was verified by flow cytometry following antibody staining with phycoerythrin-labeled UIC-2 antibody as described below.

Cytotoxicity assays

Cells were seeded in opaque white, 96-well plates at a density of 2,500 cells/well and allowed to attach overnight. Cells were then treated with increasing concentrations of the desired compound and incubated for 72 h. Cell TiterGlo (Promega) was then used to determine luminescence values for each concentration according to the manufacturer's instructions. The data were modeled using nonlinear regression curve fitting (sigmoidal, 4 parameter logistic curve model in GraphPad Prism 9 for MacOS v 9.5.1, GraphPad Software, Boston, MA) to determine the concentration at which 50% of cell growth was inhibited (GI_{50}). Where noted, cytotoxicity assays were performed with 10 μM valspodar to inhibit P-gp.

Flow cytometry assays

To measure cell surface expression of P-gp or ABCG2, trypsinized cells were incubated for 20 min. at room temperature in 2% bovine serum albumin/PBS with phycoerythrin-labeled UIC-2 antibody or phycoerythrin-labeled 5D3 antibody, respectively, according to the manufacturer's instructions (both from ThermoFisher, Grand Island, NY). Cells were also incubated with the corresponding phycoerythrin-labeled isotype control - IgG2a kappa for P-gp and IgG2b kappa for ABCG2 (both from ThermoFisher). P-gp or ABCG2 transporter activity was measured using rhodamine 123 or purpurin-18, respectively[19]. Cells were trypsinized and incubated for 30 min in complete medium (phenol red-free Richter's medium with 10% FCS and penicillin/streptomycin) with the desired fluorescent substrate (0.5 μg/mL rhodamine 123 to detect P-gp or 15 μM purpurin-18 to detect ABCG2) in the presence or absence of 25 μM concentrations of the desired FIN or a positive control inhibitor (10 μM valspodar for P-gp or 10 μM fumitremorgin C for ABCG2) for 30 min at 37 °C in 5% CO_2. Subsequently, cells were washed and incubated in substrate-free medium for 1 h at 37 °C continuing with or without inhibitor. Cells were subsequently analyzed with a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) and data analysis was performed using FloJo v 10.4.2 (FlowJo LLC, Ashland, OR).

ATPase assay

The ATPase assay was performed as described previously[20]. Total membrane vesicles were prepared from High Five insect cells overexpressing P-gp. The membranes were diluted with ATPase assay buffer (50 mM MES-Tris, pH 6.8 containing 50 mM KCl, 5 mM NaN_3, 1 mM EGTA, 10 mM $MgCl_2$, 2 mM DTT and 1 mM oubain) to reach a final concentration of 100 μg/mL and were incubated with the compounds at the noted concentrations for 10 min in the presence or absence of 0.3 mM sodium orthovanadate. The addition of 5 mM ATP (5 mM) started the reaction (20 min at 37 °C), after which SDS (2.5% final concentration) was added to terminate the reaction. The amount of inorganic phosphate released was quantified and the results were reported as a percentage of vanadate-sensitive ATPase activity with DMSO.

RESULTS

Generation and characterization of A673 cells that overexpress P-gp or ABCG2

As the A673 cell line was reported to be sensitive to FINs[4] and did not express P-gp or ABCG2, we transfected this cell line with either empty vector (A673 EV) or vectors containing the genes encoding human P-gp (A673 B1) or ABCG2 (A673 G2). We selected single clones with high levels of P-gp or ABCG2 based on measurement of antibody staining by flow cytometry and further characterized positive clones. As seen in Figure 1A, A673 B1 or A673 G2 cells were found to have much higher levels of the transporter proteins, as shown by increased staining with UIC-2 antibody or 5D3 antibody (orange histogram), respectively, compared to A673 EV cells which were negative for both transporters. P-gp-overexpressing MDR-19 cells and ABCG2-overexpressing R-5 cells served as positive controls for P-gp or ABCG2 surface expression, respectively (data not shown). Functional assays were also used to confirm transporter activity. A673 B1 cells readily transported rhodamine 123 and A673 G2 cells demonstrated increased purpurin-18 efflux, as shown by the left shift of the blue histogram for the two substrates, compared to empty vector-transfected cells (A673 EV), as illustrated in Figure 1B.

To verify that transporter levels were adequate to confer resistance to known substrates, we performed cytotoxicity assays with the P-gp substrate doxorubicin as well as the ABCG2 substrate SN-38. As seen in Figure 1C and Table 1, A673 B1 cells were resistant to doxorubicin, whereas A673 G2 cells exhibited little to no resistance to this compound. A673 G2 cells displayed increased resistance to SN-38. Having confirmed that the transfected cells expressed high levels of the desired transporters and that the transporters were indeed functional, we proceeded to use them to characterize the ability of P-gp and ABCG2 to confer resistance to FINs.

P-gp overexpression confers resistance to modified erastin derivatives

A673 EV, A673 B1 and A673 G2 cells were then used in 3-day cytotoxicity assays to determine whether the transporters could confer resistance to the FINs. While resistance due to the expression of ABCG2 or P-gp was not seen with most FINs examined, this was not true for some erastin derivatives that had been modified to improve water solubility. Overexpression of P-gp conferred relatively high levels of resistance to imidazole ketone erastin and piperazine erastin [Figure 1C], but we observed no resistance to erastin. FIN56 appeared to be a weak P-gp substrate, as A673 B1 cells were about 6-fold resistant [Table 1]. ABCG2 overexpression did not confer appreciable resistance to any of the FINs examined.

Since P-gp overexpression appeared to confer resistance to some FINs, we validated the results in parental OVCAR8 ovarian cancer cells and P-gp-overexpressing NCI/ADR-RES cells that were derived from OVCAR8 cells by selection with doxorubicin. As shown in Figure 2A, OVCAR8 cells do not express P-gp, as determined with the P-gp-specific monoclonal antibody UIC-2, while NCI/ADR-RES cells express high levels of the transporter, as shown by increased staining with the UIC-2 antibody in NCI/ADR-RES cells. Additionally, rhodamine efflux was observed in the NCI/ADR-RES cells, but not in the OVCAR8 cells. In this model system, when we performed cytotoxicity assays in the presence or absence of 10 μM valspodar, a P-gp inhibitor, we observed that P-gp overexpression conferred resistance to imidazole ketone erastin, piperazine erastin, and FIN56, and that valspodar reversed the resistance. P-gp overexpression in the NCI/ADR-RES line was not found to confer resistance to erastin or erastin2 [Figure 2B and Supplementary Table 1], in agreement with the results from the A673 cells.

Deletion of ABCB1 in UO-31 cells increases sensitivity to erastin derivatives

The renal carcinoma cell line UO-31 is known to have detectable levels of P-gp and displays rhodamine efflux[21]. To determine if the expression of P-gp in this cell line is high enough to confer resistance to imidazole ketone erastin or piperazine erastin, two of the best substrates for P-gp, we performed CRISPR-mediated deletion of *ABCB1* and selected two clones that had lost expression. As shown in Figure 3A, while

Table 1. Cross-resistance profile of transfected A673 cells[a]

Compound	A673 EV GI_{50} (μM)	A673 B1 GI_{50} (μM)	RR	A673 G2 GI_{50} (μM)	RR
SN-38	0.0018 ± 0.001	0.0024 ± 0.0004	1.3	0.042 ± 0.00013	23
Doxorubicin	0.010 ± 0.004	0.24 ± 0.075	24	0.017 ± 0.005	1.7
Erastin	2.2 ± 0.42	3.4 ± 1.2	1.5	1.9 ± 0.78	0.86
Erastin2	0.083 ± 0.004	0.17 ± 0.043	2	0.087 ± 0.024	1
Imidazole ketone erastin	0.31 ± 0.25	11.4 ± 5.4	38	0.33 ± 0.24	1.1
Piperazine erastin	1.6 ± 0.42	11 ± 2	6.9	1.4 ± 0.28	0.88
FIN56	0.70 ± 0.43	3.9 ± 2.8	5.6	0.99 ± 0.65	1.4
JKE-1674	1.0 ± 0.20	1.9 ± 0.18	1.9	2.7 ± 0.11	2.7
RSL3	0.055 ± 0.010	0.12 ± 0.008	2.2	0.06 ± 0.003	1.1
$FinO_2$	2.8 ± 1.7	2.8 ± 1.7	1	3.0 ± 1.6	1.1
PACMA31	0.047 ± 0.056	0.07 ± 0.056	1.5	0.045 ± 0.039	0.96
ML-210	0.16 ± 0.031	0.33 ± 0.028	2.1	0.20 ± 0.037	1.2
GPX4 inhibitor 26a	0.077 ± 0.036	0.14 ± 0.050	1.8	0.093 ± 0.020	1.2
ML-162	0.095 ± 0.013	0.20 ± 0.057	2.1	0.12 ± 0.026	1.3
JKE-1716	1.4 ± 0.38	2.5 ± 0.43	1.8	0.81 ± 0.054	0.58

[a]Results presented are mean GI_{50} values +/- SEM (μM). Relative resistance (RR) value was determined by dividing the GI_{50} value for A673 cells expressing a transporter by the GI_{50} value for the A673 EV cells. Three independent experiments were performed.

UO-31 cells do stain positively with the UIC-2 antibody, as shown by increased staining with the UIC-2 antibody (orange histogram), the two knockout clones, B11 and 1F4, no longer react with the antibody, as detected by flow cytometry. Additionally, we found that the knockout clones no longer efflux the P-gp substrate rhodamine 123, as shown by increased intracellular fluorescence of rhodamine 123 (blue histogram) in the clones, suggesting that the *ABCB1* gene had been deleted. The knockout clones also demonstrate increased sensitivity to the P-gp substrate romidepsin [Figure 3B]. When we performed cytotoxicity assays with imidazole ketone erastin and piperazine erastin, the knockout clones displayed increased sensitivity to both compounds by about 3- to 4-fold compared to parental cells; however, no difference in sensitivity to erastin was noted [Supplementary Table 2]. Similar results were obtained when we performed cytotoxicity assays with imidazole ketone erastin and pierazine erastin in the presence of 10 μM of the P-gp inhibitor valspodar [Supplementary Tables 1 and 3]. Thus, even relatively low levels of P-gp may cause resistance to the erastin analogs.

Ferroptosis inducers stimulate the ATPase activity of P-gp

The effect of erastin, imidazole ketone erastin, and piperazine erastin on the ATPase activity of P-gp was subsequently examined. While several P-gp substrates and some inhibitors have been shown to stimulate the ATPase activity of P-gp, not all substrates do so. We found that all three of the compounds stimulated the ATPase activity of P-gp to a degree comparable to that of verapamil, which is used as a positive control [Figure 4]. The ATPase stimulation serves as a confirmation of the interaction between imidazole ketone erastin and piperazine erastin and P-gp, given the close connection between ATP hydrolysis and substrate efflux[22]. While P-gp does not appear to confer resistance to erastin in our studies, erastin does stimulate ATPase activity. This suggests that erastin interacts with P-gp, but likely has a slow off rate, potentially acting more as a weak inhibitor than a substrate.

Ferroptosis inducers inhibit P-gp- and ABCG2-mediated transport

As targeted therapies are known to act as inhibitors of ABC transporters, we next characterized the ability of the FINs to act as inhibitors of P-gp or ABCG2. At a concentration of 10 μM, erastin2, ML-162, GPX4 inhibitor 26a, and PACMA31 inhibited P-gp-mediated rhodamine 123 transport, resulting in a 5- to 10-fold

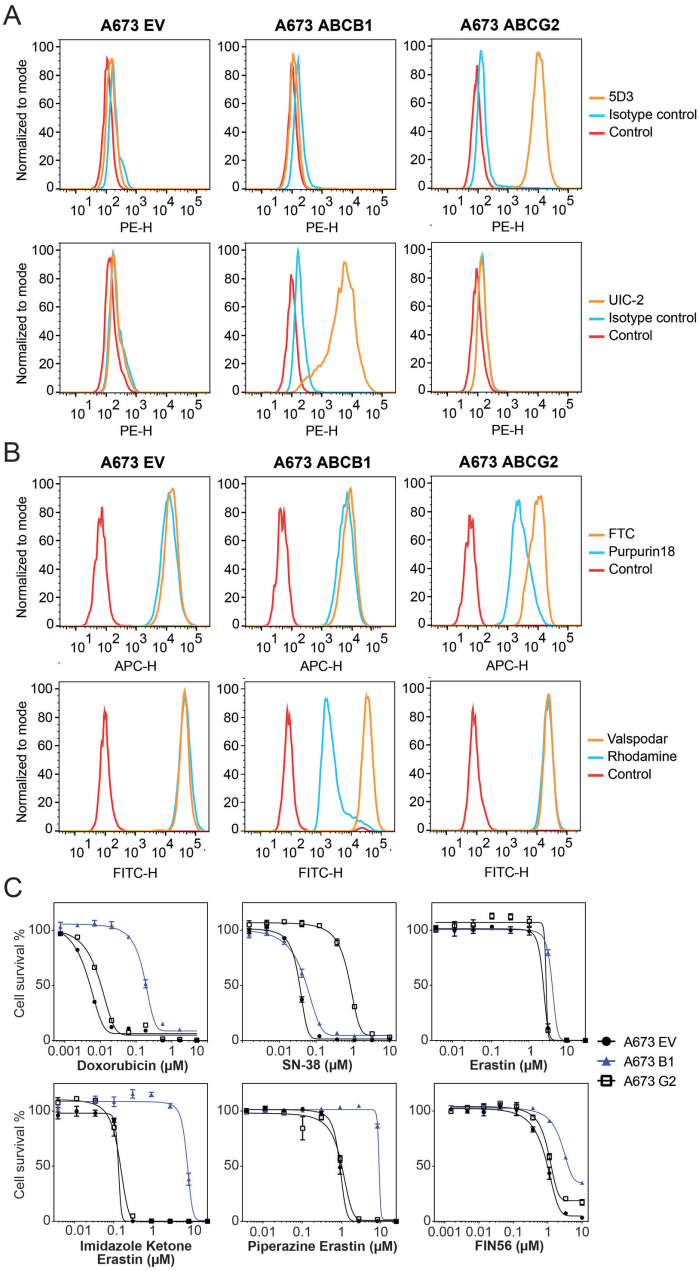

Figure 1. Characterization of A673 cells transfected to express P-gp or ABCG2. (A) Trypsinized A673 EV, B1 or G2 cells were incubated with 2% bovine serum albumin/PBS containing phycoerythrin-labeled antibody to detect ABCG2 (5D3) or P-gp (UIC-2), or the corresponding isotype control antibody for 20 min after which cells were washed in PBS. Control cells (no antibody) are denoted by red curves, isotype control staining is denoted by blue curves, and staining with specific transporter antibodies is denoted by orange curves (ABCG2 top row, P-gp bottom row). Results from one of three independent experiments are shown; (B) Trypsinized A673 EV, B1, and G2 cells were incubated with rhodamine 123 (0.5 μg/mL, for detection of P-gp) or purpurin-18 (15 μM, for detection of ABCG2) with or without appropriate inhibitor (10 μM valspodar for P-gp; 10 μM FTC for ABCG2) for 30 min, after which media was removed and replaced with substrate-free medium continuing with or without inhibitor for an additional 1 h. Cell autofluorescence (control) is denoted by red histograms, substrate efflux is denoted by blue histograms and cells with substrate and inhibitor are denoted by orange histograms. Results from one of three independent experiments are shown; (C) Three-day cytotoxicity assays were performed on A673 EV, B1, and G2 cells with doxorubicin, SN-38, erastin, imidazole ketone erastin, piperazine erastin and FIN56. Results from one of three independent experiments are shown and results are summarized in Table 1.

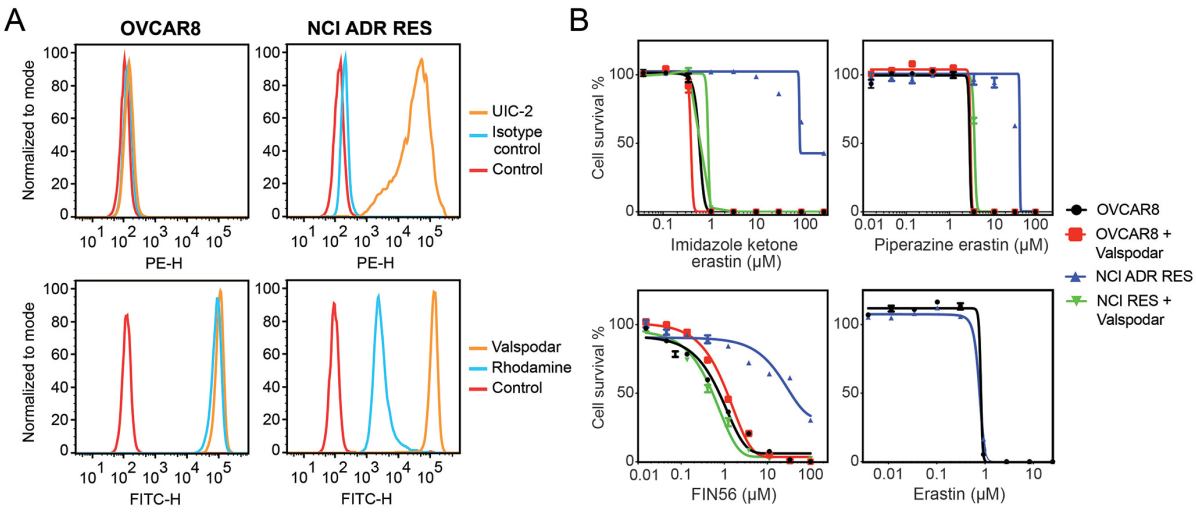

Figure 2. The OVCAR8 and NCI/ADR-RES cell line pair confirm P-gp substrates. (A) Trypsinized OVCAR8 or NCI/ADR-RES cells were incubated with 2% bovine serum albumin/PBS containing phycoerythrin-labeled UIC-2 antibody to isotype control antibody for 20 min, after which cells were washed in PBS and read on a flow cytometer. Control cells (no antibody) are denoted by red curves, isotype control staining is denoted by blue curves, and P-gp staining is denoted by orange curves (ABCG2 top row, P-gp bottom row). Results from one of three independent experiments are shown; (B) Three-day cytotoxicity assays were performed on OVCAR8 and NCI/ADR-RES cells with imidazole ketone erastin, piperazine erastin, FIN56 or erastin. Where noted, the P-gp inhibitor valspodar was added at a concentration of 10 μM. Results from one of three independent experiments are shown and results are summarized in Supplementary Table 1.

increase in rhodamine fluorescence in MDR-19 cells [Figure 5A]. In ABCG2-expressing R-5 cells, GPX inhibitor 26a had the greatest effect on purpurin-18 efflux, while piperazine erastin, ML-162, PACMA31 and RSL3 also significantly inhibited purpurin-18 efflux [Figure 5B]. These results suggest that drug-drug interactions might occur during treatment with FINs.

DISCUSSION

Ferroptosis induction by small molecules is a novel way to induce cell death in cancer cells and several cancer cell types are sensitive to ferroptosis induction, such as renal cell carcinoma, diffuse large B-cell lymphomas, as well as many chemotherapy-resistant cancer subtypes[4,23]. FINs are effective in xenograft mouse models and have been suggested as potential cancer treatments[6]. Despite the recent proliferation of papers describing novel molecules that can induce ferroptosis[9-11,24], very few studies have addressed potential interactions with ABC transporters that might limit bioavailability or brain penetration. In our study, we found that the FINs FIN56, imidazole ketone erastin and piperazine erastin are transported by P-gp, suggesting that their oral bioavailability and/or brain penetration may be compromised. Additionally, we found that the FINs ML-162, GPX inhibitor 26a, and PACMA31 act as inhibitors of P-gp. Interestingly, the most potent P-gp inhibitor, GPX inhibitor 26a, was also the most potent ABCG2 inhibitor, suggesting that treatment with FINs may cause drug-drug interactions.

Our findings regarding erastin differ from those of Zhou *et al.* who reported erastin as a P-gp substrate[25]. However, we note that they used a P-gp-expressing cell line that was generated by gradually increasing exposure to paclitaxel[25]. While selection with paclitaxel can lead to P-gp overexpression, other mechanisms of resistance can arise[26]. It cannot be ruled out that other mechanisms besides efflux by P-gp may have caused the resistance to erastin observed by Zhou *et al.* Unfortunately, they did not perform cytotoxicity assays in the presence of a P-gp inhibitor, which would have confirmed the role of P-gp. In contrast, we did not observe erastin resistance in cells that were transfected to express P-gp without selection with an

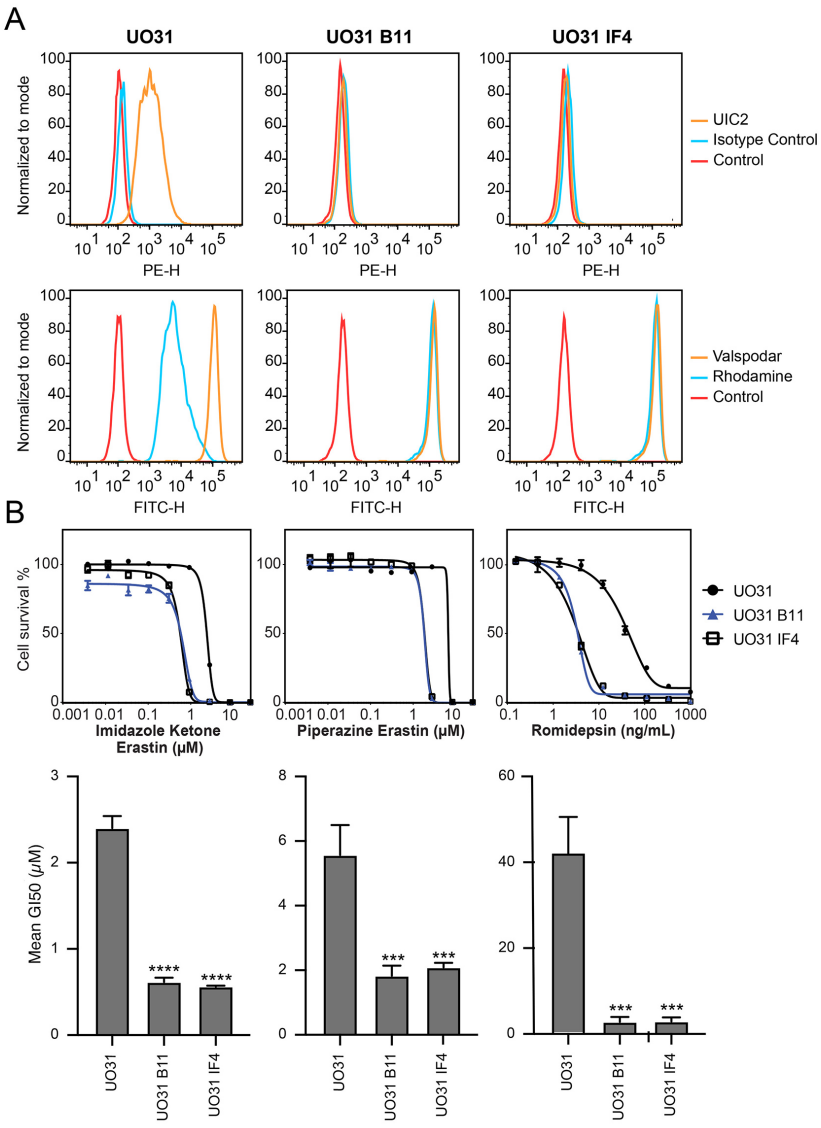

Figure 3. CRISPR-mediated deletion of *ABCB1* sensitizes UO-31 cells to FINs. (A) Top row: UO-31 cells or the *ABCB1* knockout clones (B11, 1F4) were trypsinized and incubated with 2% bovine serum albumin/PBS containing phycoerythrin-labeled UIC-2 antibody or isotype control antibody for 20 min after which cells were washed in PBS and read on a flow cytometer. Control cells (no antibody) are denoted by red curves, isotype control staining is denoted by blue curves, and staining with UIC-2 is denoted by orange curves. Bottom row: Cells were incubated with rhodamine 123 (0.5 μg/mL) with or without 10 μM valspodar for 30 min, after which media was removed and replaced with substrate-free medium continuing with or without inhibitor for an additional 1 h. Cell autofluorescence (control) is denoted by red histograms, rhodamine efflux by blue histograms, and cells with rhodamine and inhibitor are denoted by orange histograms. Results from one of three independent experiments are shown; (B) Three-day cytotoxicity assays were performed on UO-31 cells or the *ABCB1* knockout clones with romidepsin, imidazole ketone erastin or piperazine erastin. Results from one of three independent experiments are shown. GI_{50} values from 3 independent experiments are shown under the representative graphs. Significance was determined by a one-way ANOVA followed by a Dunnett test for multiple comparisons. Asterisks denote significant differences from the parental UO-31 cell line, where ***$P < 0.001$ or ****$P < 0.0001$.

anticancer drug, and this result was confirmed in a selected cell line. We thus conclude that erastin is not a P-gp substrate and that the cell line used by Zhou *et al.* may have another mechanism at work that can confer resistance to erastin[25].

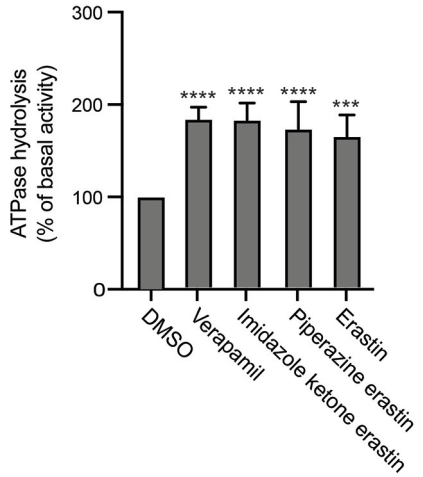

Figure 4. Effect of erastin derivatives on ATPase activity of P-gp. The effect of imidazole ketone erastin, piperazine erastin and erastin or the vanadate-sensitive ATPase activity of P-gp was determined as outlined in Materials and Methods. Basal ATPase activity was compared to that in the presence of 10 μM concentrations of the compounds; verapamil at 10 μM served as a positive control for stimulation of ATPase activity. Significance was determined by a one-way ANOVA followed by a Dunnett test for multiple comparisons. Asterisks denote significant differences from the DMSO control, where ***$P < 0.001$ or ****$P < 0.0001$.

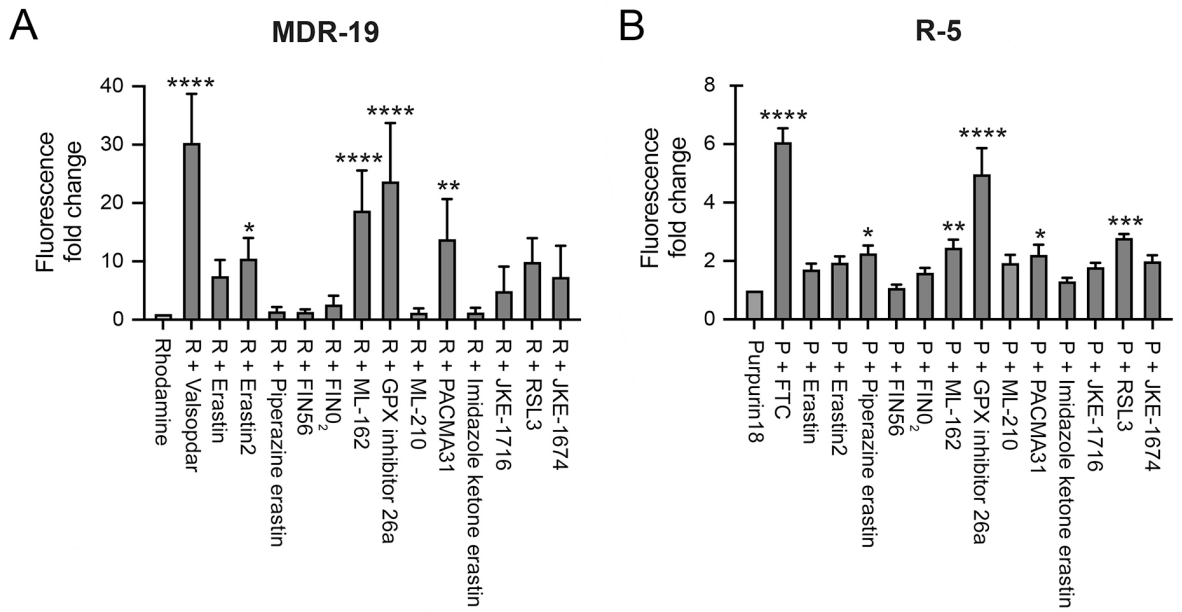

Figure 5. FINs inhibit P-gp and ABCG2 transport activity. P-gp-overexpressing MDR-19 cells (A) or ABCG2-overexpressing R-5 cells (B) were incubated with 0.5 μg/mL rhodamine 123 or 15 μM purpurin-18, respectively, in the absence or presence of specific inhibitor (10 μM valspodar for P-gp and 10 μM FTC for ABCG2) or 10 μM concentrations of the FINs for 30 min after which the medium was removed and replaced with substrate-free medium with or without the inhibitor. Cells were then incubated for an additional 1 h. Inhibition of P-gp or ABCG2 was determined by calculating the fold increase in intracellular fluorescence, with fluorescence levels in cells incubated with rhodamine or purpurin-18 alone assigned a value of 1. Significance was determined from three independent experiments using a one-way ANOVA followed by a Dunnett test for multiple comparisons. Asterisks denote significant difference from the rhodamine or purpurin-18 control, where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, or ****$P < 0.0001$. P: Purpurin-18; R: rhodamine.

It is not surprising that the FINs, which are essentially targeted therapies, interact with drug transporters, as many targeted therapies have been shown to either be substrates or inhibitors of P-gp or ABCG2[27]. The

BCR-ABL inhibitors imatinib, nilotinib, dasatinib, and bosutinib have been found to be substrates of P-gp and ABCG2 at low concentrations, while they act as inhibitors of the proteins at higher concentrations[28-31]. Overexpression of *ABCB1* has been demonstrated in tumor samples obtained from patients whose tumors have developed resistance to the ALK inhibitor ceritinib in the absence of secondary ALK mutations[32]. Ceritinib has also been reported to inhibit P-gp- and ABCG2-mediated transport[33]. Additionally, both P-gp and ABCG2 have been shown to confer resistance to several structurally different aurora kinase inhibitors[34,35].

The ability of P-gp and ABCG2 to affect brain penetration of targeted therapies has most dramatically been demonstrated in mouse models in which the *ABCB1* homologs *Abcb1a* and *Abcb1b* are knocked out, the ABCG2 homolog *Abcg2* is knocked out, or all of the homologous transporters have been deleted. Brain concentrations of the Janus kinase 1/2 inhibitor momelotinib 24 h after oral administration were 6.5-fold, 3-fold and 48-fold higher in mice deficient in Abcg2, Abcb1a/b, or Abcg2;Abcb1a/b, respectively, compared to control mice[36]. Similarly, 24 h after oral administration of the BCR-ABL inhibitor ponatinib, brain concentrations were 2.2-fold, 1.9-fold and 25.5-fold higher in mice deficient in Abcg2, Abcb1a/b, or Abcg2;Abcb1a/b, respectively, compared to wild-type controls[37]. Thus, P-gp and ABCG2 can have a profound effect of limiting brain penetration of targeted therapies that are substrates of the transporters. Brain accumulation or oral bioavailability of FIN56, imidazole ketone erastin or piperazine erastin could similarly be affected, as they were found to be transported by P-gp.

In conclusion, we have demonstrated that the FINs FIN56, imidazole ketone erastin, and piperazine erastin are substrates of P-gp, suggesting a potential reduction in oral bioavailability and brain penetration. ML-162, GPX inhibitor 26a and PACMA31 were found to inhibit the transport activity of P-gp and/or ABCG2, suggesting potential drug-drug interactions. Thus, our findings will be valuable as these compounds are pursued clinically.

DECLARATIONS

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Wrote or contributed to the writing of the manuscript: Frye WJE, Huff LM, Salazar P, Robey RW, Ambudkar SV, Gottesman MM

Availability of data and materials

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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The multifaceted role of extracellular vesicles in prostate cancer-a review

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Abstract

Prostate cancer is the second most prominent form of cancer in men and confers the highest mortality after lung cancer. The term "extracellular vesicles" refers to minute endosomal-derived membrane microvesicles and it was demonstrated that extracellular vesicles affect the environment in which tumors originate. Extracellular vesicles' involvement is also established in the development of drug resistance, angiogenesis, stemness, and radioresistance in various cancers including prostate cancer. Extracellular vesicles influence the general environment, processes, and growth of prostate cancer and can be a potential area that offers a significant lead in prostate cancer therapy. In this review, we have elaborated on the multifaceted role of extracellular vesicles in various processes involved in the development of prostate cancer, and their multitude of applications in the diagnosis and treatment of prostate cancer through the encapsulation of various bioactives.

Keywords: Extracellular vesicles, prostate cancer, stemness, chemoresistance, therapeutic delivery, diagnosis

INTRODUCTION

One of the most common forms of cancer identified in men is prostate cancer (PCa), which is also one of the deadliest, the second most common type of cancer in men after lung cancer, and the fifth most prevalent

mortality cause in males[1]. In the United States of America alone in 2021, more than 248,000 instances of PCa were identified in men, and just under 34,000 fatalities were attributed to the disease[2]. Over 1.4 million cases worldwide were reported, with more than 300,000 deaths related to PCa[3]. Currently, multiple treatment strategies are used against PCa. These therapies include surgery, radiation therapy, active surveillance, and proton therapy. Apart from these, other strategies including chemotherapy, hormonal therapy, cryosurgery, and HIFU (high-intensity focused ultrasound) are also part of the current treatment regime based on the clinical conditions and outcomes[4-10]. Active surveillance emerged as the management option for the low-risk PCa and various strategies were developed for the same[11]. Urological guidelines proposed various treatment options depending on the stage of PCa. For example, patients with low-risk diseases are offered active surveillance and active treatment such as surgery or radiation therapy. For immediate-risk disease patients, radical prostatectomy, radiotherapeutic therapy, and pelvic lymph node dissection is the treatment option. The same treatment option applies to patients with high-risk localized disease. In addition, radiotherapy and surgery are used as a treatment for locally advanced disease[12]. Hormonal therapy, also known as androgen deprivation therapy (ADT), is a traditional and standard therapy against PCa for over 60 years and shows its effect through the decrease in serum testosterone[13]. Hormonal therapies have also shown combinative strategies with other standard treatment options such as radiotherapy, non-invasive treatments through medications, and hormonal therapy with surgery[14,15]. Hormonal therapy with drugs in prostate cancer provides effective tumor growth and progression control, boosting patient outcomes and quality of life. At present, commonly used medications in hormonal therapy involve drugs such as enzalutamide, abiraterone, goserelin, bicalutamide, *etc.*[16-21].

Following hormonal therapy for a while, the tumor develops into metastatic castration-resistant PCa (mCRPC), which has a low rate of survival and few treatment options[22-24]. The manner in which multilaminar bodies, particularly extracellular vesicles, influence the general environment, the processes, and the expansion of PCa cells is one of the areas that has demonstrated a significant amount of potential when it comes to the treatment of PCa.

The term "extracellular vesicles" was primarily used by Rose Johnstone and her colleagues in 1970 to refer to minute endosomal-derived membrane microvesicles[25,26]. Extracellular vesicles offer several advantages, such as the ease of therapeutic cargo loading (drugs, siRNA), the ability to penetrate through biological barriers, low immunogenicity, ease of cellular uptake, ease of surface modification, *etc.*[27]. However, limitations such as difficulty in obtaining high quantities of pure extracellular vesicles, purification process, and disadvantages pertaining to various isolation techniques limit the application of extracellular vesicles in drug delivery. The advantages and disadvantages associated with extracellular vesicles are graphically depicted in Figure 1[28].

Substantial numbers of researchers concentrated their attention on the roles of extracellular vesicles in the act of tumors. There is a lot of interest in how extracellular vesicles affect the environment in which tumors originate, the role that extracellular vesicles play in the progression of drug resistance in cancer cells in response to anti-cancer medications, and the role that extracellular vesicles can play in mitigating the effects of cancer and perhaps halting the process of cancer growth as well as their potentially intricate role in the delivery of chemotherapeutic agents and other bioactives in the tumor cells. Extracellular vesicles, which initially were considered molecular waste bins, now have shown a multifaceted role in cancer. Extracellular vesicles serve a significant function in angiogenesis through the transport of various pro-angiogenic biomolecules, such as vascular endothelial growth factor (VEGF), microRNAs, and matrix metalloproteinases (MMPs), and also act as key agents in metastasis through their involvement in restructuring metastatic sites to support cancer cell colonization[29-31]; Extracellular vesicles have a key role as

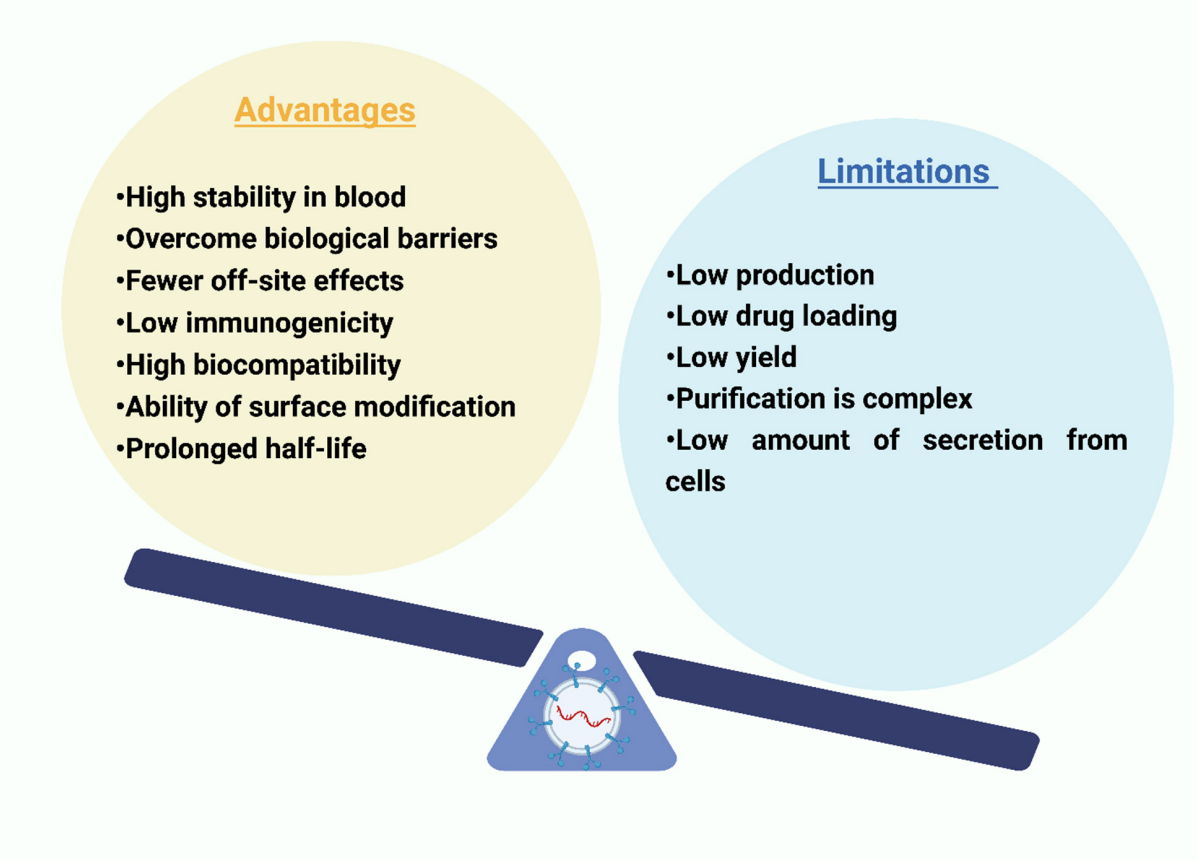

Figure 1. Graphical representation of advantages and limitations of extracellular vesicles.

biomarkers in the cancer development through its components including proteins, nucleic acids and also from other biofluids [Table 1][32]. While talking about the exosomal role in PCa, they have been indulged in acting as the key agent in the progression of PCa through numerous mechanisms including changes in the tumor microenvironment and angiogenesis, through the metastasis and cell proliferation, and in the drug resistance in PCa tumor[33-43].

In this review article, we have described the multifaceted functions that extracellular vesicles play in PCa. This review focuses on the pro-tumorigenic role of extracellular vesicles in the key processes engaged in the development of PCa, such as stemness, chemoresistance, radioresistance, angiogenesis, and metastasis. Furthermore, the application of extracellular vesicles in the diagnosis and delivery of therapeutic moieties such as the siRNA, drugs, and phytoconstituents are also elaborated in this review.

PRO-TUMORIGENIC ROLE OF EXTRACELLULAR VESICLES IN PCa

Role of extracellular vesicles in stemness of PCa

Stemness refers to the phenomenon that defines the cells' capability of self-renewal and differentiation[61]. It is a characteristic ability shown by adult stem cells to proliferate, and bolster their new generation of daughter cells and also interact with their environment to maintain a balance between quiescence, proliferation, and regeneration[62]. In the case of cancer stem cells (CSCs), this phenomenon acts as a malignant equivalent to normal stem cells. Apart from the common features such as maintenance, sustainability, supporting the microenvironment, and self-renewal, the essential differences found in the

Table 1. List of biomarkers from extracellular vesicles in prostate cancer

Biomarkers from extracellular vesicles		The role played in prostate cancer	Cell line/ model used	References
Proteins	HSP70	Provides drug resistance, promotes invasion, stemness, and metastasis	PC3 and LNCaP	[44-46]
	Caveolin 1 (CAV1)	Progression and metastasis of Prostate cancer; inhibit apoptosis in prostate cancer cells.	LNCaP and PC3 cell lines	[47,48]
	Integrin alpha-2 (ITGA2)	Mediates cancer progression and metastasis; possible role in alteration of AR phenotype and development of aggressive prostate cancer	CRPC-derived extracellular vesicles, LNCaP cell lines	[49,50]
	Annexin A2	Enhances the release of IL-6, promoting the proliferation of prostate cancer; migration and adhesion to osteoblasts	DU145, LNCaP, PC-3	[51-53]
	Vimentin	Associated with invasion and metastasis via Src regulation	PC-3M-1E8 PC-3M-2B4	[54]
RNA	miR-21	Promotes growth as well as proliferation following the surgical castration; Also promotes invasion and apoptosis resistance	LNCaP, androgen-dependent PC-3 cell lines, and DU-145 cell lines	[55,56]
	TMPRSS2-ERG fusion	Promotes cell proliferation	TMPRSS2-ERG-positive PCa xenograft models	[57]
	miRNA-221/222	Promotes oncogenesis and progression of prostate cancer through p27(Kip1) downregulation	PC-3(aggressive prostate carcinoma model), LNCaP, and 22Rv1 cell line models	[58]
	MALAT1	Through sponging miR-145 promotes cell proliferation, migration, and invasion	LNCaP and CWR22Rv1 cell lines	[59]
	HOTAIR (HOX transcript antisense RNA)	Decreasing the inhibitory effect of hepaCAM on MAPK signaling promotes invasion and metastasis.	Samples collected from patients at Dept. of Urology, First Affiliated Hospital of Chongqing Medical University, China	[60]

cancer stem cells refer to extra features that include heterogeneity in the cell population, high resistance towards hostile factors like quiescence, chemotherapeutic agents, hypoxia and low nutrient levels[62-67].

Stemness plays an influential role in the development of PCa. The prostate stem cells reside in the basal and luminal layers and are a major target for the oncogenic transformation, which suggests a role in the genesis of PCa[68]. CSCs demonstrated an altered gene expression in exposure to hypoxia, nutrient deficiency, and oxidative stress, rendering them more mobile, invasive, and resilient to further stress. CSCs are anticipated to have endured epithelial-mesenchymal transition + , and the transition to mesenchymal marker expression is frequently one measurement of PCa progression. CSCs are predicted to invade locally and then metastasize[69].

Extracellular vesicles portray a substantial part in the stemness of PCa. They have been shown to be involved in multiple features exhibited by PCa stem cells, such as resistance against hypoxia and tumor progression, promotion of epithelial-to-mesenchymal transition (EMT), and also the transformation of other stem cells into cancer stem cells. Ramteke *et al.* in their work discovered that extracellular vesicles derived from hypoxic PCa cells promote the cancer-associated fibroblast (CAF) phenotype in prostate stromal cells, and also elevate the property of stemness and protruding of naive PCA (PCa) cells. They subjected human PCA PC3 and LNCaP cells to normoxic and hypoxic conditions, respectively, and extracted the extracellular vesicles released in both situations. They observed increased amounts of Annexin II, heat shock proteins (HSP90 and HSP70), and tetraspanins (CD63 and CD81) in hypoxic extracellular vesicles, which led to LNCaP and PC3 cell invasiveness and motility. Aside from that, they discover an increased amount of metalloproteins as well as increased levels of various signaling molecules. Further, in proteome analysis, they found an increased number of proteins in hypoxic extracellular vesicles, which promotes epithelial adheres junction pathway remodeling and proteins, especially in naïve PC3 cells. This study overall suggests how extracellular vesicles promote the stemness of PCa cells, affecting significant

features such as invasiveness and tumor microenvironment, leading to aggressive PCa[44]. In another study, it was found how extracellular vesicles containing PSGR (Prostate-specific G-protein coupled receptor) promote the migration, invasiveness, and stemness of low-aggressive PCa cells. They used transcriptome sequencing to determine the differentially expressed (DE) mRNAs in low invasive cells incubated with overly expressed PC3 extracellular vesicles or negative control (NC) extracellular vesicles. They also discovered that the PSGR was stably overexpressed in PC3 cells. Internalization of PC3 PSGR + extracellular vesicles in LNCaP and RWPE-1 cells greatly promoted cell migration and invasion. After PC3 PSGR + exosome incubation, E-cadherin expression declined, while vimentin, Snail, SOX2, and OCT4a expression elevated in low invasive cells. This resulted in findings indicating that extracellular vesicles released via PCa cells induce invasiveness and stemness[70]. An interesting study was published talking about how extracellular vesicles derived from PCa cells promoted the neoplastic reprogramming of adipose stem cells derived from the patient. They identified that extracellular vesicles obtained from PCa facilitated the transformation of adipose stem cells into neoplastic cells in the patient due to changes in the cell microenvironment. They observed that pASCs (PCa patients derived adipose-stem cells) primed with PCa cell conditioned media (CM) produced prostate-like neoplastic lesions in vivo and replicated aggressive tumors in secondary recipients, in contrast to normal ASCs (adipose-stem cells). The cytogenetic aberrations and mesenchymal-to-epithelial transition of the pASC tumors, along with the expression of epithelial, neoplastic, and vasculogenic markers, were evocative of molecular features of PCa tumor xenografts. This suggests that extracellular vesicles play a tremendous role in not only promoting the stemness, invasiveness, and growth of PCa cells but can also be an agent advocating the transformation of other stem cells into oncogenic cells[71-73].

Extracellular vesicles in drug resistance and radioresistance of PCa

Extracellular vesicles and drug resistance

The development of resistance to treatment drugs has been an extremely difficult obstacle to overcome in PCa treatment[43,74]. The use of chemotherapy is still considered to be one of the more traditional methods for treating advanced PCa[5]. However, it has been demonstrated that several variables, including the heterogeneity of the tumor, epigenetic control by miRNAs, and the combinatorial outcomes of various signaling pathways, including NF-κB/IL-6, Hedgehog, mTOR (mammalian target of rapamycin), Akt/PI3K, MAPK/ERK, and somatostatin receptors, all of these elements result in drug resistance in tumor cells[75-79].

According to numerous research, extracellular vesicles are involved in the development of medication resistance in PCa[42,74,80]. Extracellular vesicles have been discovered as the mechanism underlying resistance against the drug enzalutamide, and in PCa, the emergence of therapy-induced neuroendocrine differentiation stages, as demonstrated by Bhagirath *et al.* in their research[74]. In addition, it was observed that BRN2 and BRN4, which are neural transcription factors, were liberated in PCa extracellular vesicles after the treatment with enzalutamide. These transcription factors are essential for the neuroendocrine remodeling of prostate adenocarcinomas[74,81,82]. Kharaziha *et al.* discovered with the usage of nanoparticle tracking analysis that a greater quantity of extracellular vesicles released by DU145 PCa cells docetaxel-resistant than by DU145 PCa docetaxel-sensitive cells[80]. Extracellular vesicles were shown to be responsible for resistance against docetaxel in docetaxel-susceptible PCa cells (DU145, LNCaP, and 22Rv1)[42]. Following administration of the cells with docetaxel-resistant extracellular vesicles versions of 22Rv1 and DU145 (22Rv1RD and DU145RD, respectively), authors observed the liberation of MDR-1/Pgp, which is a multidrug resistance protein 1/P-glycoprotein and this transporter protein engaged in the efflux of a wide range of exogenous objects, including antineoplastic medications, from extracellular vesicles, played a probable role in imparting resistance to docetaxel-susceptible cells[42]. Another study looked through an interesting pathway to study the chemoresistance in PCa cells. In the study, exosome-derived miR-27a, which portrays a substantial role in chemoresistance in cells of PCa, was examined. When administered

with doxorubicin, cisplatin, and docetaxel in PCa cells, there was a tremendous spike in the levels of the miR-27a; they also co-treated PC3 cells (PCa cells) with primary prostate fibroblasts (PSC27 cells) to analyze tumor treatment resistance mechanisms. The results additionally demonstrate that exosome-derived miR-27a produced by PSC-27 cells enhanced chemoresistance by suppressing P53 gene expression[35]. Although it is not the commonly observed pathway for drug resistance, Saari *et al.* in their research made an interesting discovery. They observed that using two contrasting populations of extracellular vesicles (microvesicle- and exosome-enriched) as paclitaxel carriers in autologous PCa increased the cytotoxicity effect of the paclitaxel, regardless of the fact that cancer cell viability increased without the vesicles, but the overall net cytotoxicity effect remained increased. They also observed that this phenomenon was irrespective of the EV population and cell lines tested[83].

In recent years, various studies demonstrated the role played by the extracellular vesicles in the development of drug resistance in PCa. Shan *et al.* observed that miRNA-423-5p from extracellular vesicles, which was secreted from cancer-associated fibroblasts, was promoting chemoresistance in prostate cancer. They observed that miRNA-423-5p from extracellular vesicles promoted chemoresistance through inhibition of GREM2 via the TGF-β pathway for taxane derivatives while suppressing the TGF-β pathway was able to partially reverse the chemoresistance[84]. In another study, Kato *et al.* utilized the serum extracellular vesicles containing CD44v8-10 mRNA as the diagnostic marker for docetaxel resistance in prostate cancer. The results showed that the levels of CD44v8-10 protein and mRNA in cell lysates and extracellular vesicles were higher in PC-3R cells, which was a docetaxel-resistant cell line compared to normal PC-3 cells. This showed that CD44v8-10 protein had a role in the development of chemoresistance[85]. The role of syntaxin-6-mediated extracellular vesicles in the regulation of enzalutamide resistance in prostate cancer was demonstrated. The authors observed that the enzalutamide-resistant cell lines (CWR-R1, C4-2B, and LNCaP) had a higher amount of extracellular vesicle secretion (about 2-4) times than the enzalutamide-sensitive cells. The observed mechanism underlying was found to be the upregulation of syntaxin-6 accompanied by the increase in colocalization with CD-63 in enzalutamide resistance cell lines. They also observed that knocking down syntaxin-6 with siRNAs resulted in a reduction in cell count and an enhancement in cell death in the presence of enzalutamide[86].

Extracellular vesicles and radioresistance

Apart from the drug resistance shown in PCa in the above section, extracellular vesicles are also associated with radioresistance in PCa[87]. In the case of PCa, following the radiation therapy, increased levels of HSP72-containing extracellular vesicles were found in PCa, leading to the conclusion that HSP72-containing extracellular vesicles are a possible patron, leading to pro-inflammatory cytokine production and immune modulation[88]. Other than this, another phenomenon was observed in the cancer stem cells that tend to show more resistance against radiation therapy. This allows the production of extracellular vesicles that will deliver the resistance phenotype to recipient cells[87]. This will allow for the formation of more resistant cancer cells to radiation therapy. Regarding PCa, research has demonstrated the presence of extracellular vesicles released from prostate stem cells; these vesicles have the capacity for autophagy and can also modulate the sensitivity towards radiation therapy[89,90]. All of this suggests a strong negative role of extracellular vesicles not only as a key agent against drug and chemoresistance, but extracellular vesicles also play a quintessential role in the emergence of resistance against radiation therapy in PCa[91]. Radiation therapy, still one of the mainstream therapies for the treatment of cancer across all types, can affect the release of content from extracellular vesicles, which has already been affected by the composition and abundance of the extracellular vesicles, affecting the extracellular vesicle-based intracellular communication.

Extracellular vesicles in metastasis of PCa

Aside from their influence at metastatic locations, tumor-derived extracellular vesicles have been shown to have a role in the invasion, development, and metastasis of tumors by interacting with cells at isolated, pre-metastatic organ locations[92,93]. This transpires through the establishment of tumor-nurturing microenvironments, a technique known as "pre-metastatic niche generation". The formation requires protracted intercellular communication facilitated by soluble or membrane-bound proteins originating from the original tumor[94-96].

The tumor-originated extracellular vesicles are responsible for the extracellular matrix (ECM) remodeling via the accumulation of fibronectin and the promotion of crosslinking by the ECM-modifying enzyme lysyl oxidase (LOX). This is done with the intention of improving bone marrow-derived cell adherence, which is a critical component of the pre-metastatic niche. To improve the adherence of bone-marrow-derived cells, tumor-originated extracellular vesicles remodel the ECM through the aggregation of fibronectin and crosslinking ECM[97-99].

Intriguingly, new research conducted by Henrich *et al.* indicates convincingly that homeostasis of cholesterol in bone marrow myeloid cells is the mediator of communication between bone marrow and PCa cells through extracellular vesicles. They observed that bone marrow myeloid cells absorb PCa extracellular vesicles, leading to an activated NF-κB signaling, improved osteoclast formation *in vitro* and *in vivo*, and lessened myeloid thrombospondin-1 expression[100]. A tailored biomimetic strategy involving myeloid cells *in vitro* and *in vivo* lowering cholesterol levels prevented PCa EV uptake by recipient myeloid cells, eliminated NF-κB activity, maintained thrombospondin-1 expression, decreased osteoclast differentiation, and yielded a 77% reduction in metastatic burden[100]. During early stages of metastasis, the EMT is a pivotal critical step. This state is triggered by tumor-derived extracellular vesicles' autocrine and paracrine signaling and targeting proteins such as transforming growth factor-beta (TGF-β) and catenin, which are EMT-related proteins[101,102]. Extracellular vesicles derived from PCa DU145 and PC3 cells generate a type of TGF-β capable of initiating fibroblast-myofibroblast transformation by activating the TGF-SMAD signaling pathway. This TGF-β is also capable of promoting tumorigenesis and inhibiting immune response[103,104]. McAtee *et al.* showed in their research that prostate tumor cells containing extracellular vesicles have a protein called hyaluronidase 1 (Hyal1) in them. This protein encourages the relocation of prostate stromal cells, which ultimately speeds up the evolution of PCa[105]. Research conducted by Josson *et al.* showed that the microRNA miR-409, which has a crucial part in the EMT in PCa, was observed in stromal-derived extracellular vesicles[106]. Furthermore, integrins are known to play a role in metastasis and progression of cancer, and in PCa, the $\alpha_v\beta_6$ integrin is shown to enhance the ab

ility to migrate[107]. Furthermore, the integrins derived from extracellular vesicles are also known to play a role in angiogenesis[108]. In a research study, the proteins Integrin Subunit Alpha 3 (ITGA3) and Integrin Subunit Beta 1 (ITGB1) were found in extracellular vesicles recovered from LNCaP and PC3 cells, released in the urine of PCa patients, both of them contributing to the diaspora and invasion of both tumors[109]. It is worth noting that Elmageed *et al.* reported that exosome-derived miR-130b, miR-125b, and miR-155 promote mesenchymal-epithelial transition (MET) and neoplastic transformation and stem cells isolated from PCa patients' adipose tissue[71]. Honeywell *et al.* illustrated in their research how miR-105 obtained from tumor-derived extracellular vesicles serves as a tumor suppressor and targets the cyclin-dependent kinase 6 (CDK6), which suppresses the cell proliferation[110]. Another interesting route was discovered, bolstering a pathway for metastasis in PCa. It was also demonstrated that androgen receptors were expressed by PCa cells-derived extracellular vesicles, which, after nuclear localization, led to enhanced cell proliferation[111]. Metastasis of lymph nodes to distant organs can be observed through the probable mediation of extracellular vesicles. Even though a mystery is shrouded suggesting the initiation of this

process by which tumor cells, it is possible that lymph node metastasis could occur through extracellular vesicles. The authors of the research, Maolake *et al.*, revealed that the tumor necrosis factor-α (TNF-α) provides a huge contribution towards metastasizing PCa lymph nodes using LNCaP, DU145, LNCaP-SF, and PC3 cell lines of PCa can be seen through the activation of chemokine (C-C motif) ligand 21/CC chemokine receptor 7 (CCL21/CCR7) axis and this discovery may mediate the influence that extracellular vesicles have on metastasis through the lymphatic pathway[112]. Furthermore, in another research conducted, it was discovered that from the PC3, DU145, CWR-R1 PCa, and LNCaP cells, which were derived from designated metastatic PCa cell lines, integrin alpha 2 subunits (ITGA2) were found packaged in extracellular vesicles. The fact that inhibiting the release of extracellular vesicles to recipient cells in these metastatic PCa cells by knocking down ITGA2 revealed a possible function for ITGA2 in contributing to the development of the illness and the aggressive phenotypes found in PCa. With an elevated Gleason Score of 9, lymph node metastatic tissues were found to have higher ITGA2 expression than lower Gleason Score of 7 PCa tissues, implying that ITGA2 may play a role in enhancing lymph node metastasis[49]. Furthermore, no notable differences were observed in ITGA2 protein expression levels between 24 primary PCa tissues and their matched metastatic lymph node tissues. Regardless of the fact that there were no key differences, this was the case.

Role in progression and angiogenesis

Extracellular vesicles are released constitutively by tumor cells of diverse sources. These extracellular vesicles play essential roles in the transformation of tumors into malignant forms and the progression of cancers. Extracellular vesicles' effects are mediated by the transfer of cargo, which includes a range of proteins as well as RNA (including miRNAs) and DNA[113,114]. Cancer cells essentially require to sustain their attributes, such as immortality, continued proliferative activity, evasion of immune reactions, angiogenesis, continued invasion and metastasis, and resistance to cell death or apoptosis. The tumor microenvironment supplies this medium, which includes tumor stromal cells such as mesenchymal stromal cells, fibroblasts, pericytes, and immune cells such as T & B lymphocytes. They serve as the growth medium for the tumor cells, which are located within the tumor. The transformation of normal stroma cells into reactive stroma cells, which promotes the growth of cancer cells as well as metastasis, is a feature that is distinctive of the progression of cancer. After being impacted, the stromal cells will employ extracellular vesicles to regulate the microenvironment of the tumor, therefore promoting both the development of the tumor and its ability to metastasize[115-117]. TME extracellular vesicles now perform the role of cellular communicators in addition to assisting with a range of activities. In the instance of PCa, DeRita *et al.* discovered that protein Src was found in PCa cell extracellular vesicles. Through integrin activation, this protein activates focal adhesion kinase, which leads to angiogenesis and metastasis[34]. Eventually, tumor cells and the stromal cells that are in close vicinity will engage in a persistent kind of interaction[115]. By encouraging the differentiation of fibroblasts into a myofibroblast-like phenotype that produces angiogenesis and tumor development, cancer extracellular vesicles that contain TGF- β play critical roles in the generation of stroma that promotes the growth of tumors[30,118]. The TGF-β/SMAD3 cascade has to be activated to produce this effect. The extracellular vesicles that are produced by PCa cells have a high level of latent TGF-β expression. Through focal adhesion kinase via integrin activation, this protein triggers metastasis and angiogenesis[119,120].

The production of extracellular vesicles by PCa cells enables the delivery of sphingomyelin and CD147 into endothelial cells, which facilitates cancer cell migration and endothelial cell pro-angiogenic activity[121-122]. Proteins such as c-Src tyrosine kinase, IGF-R (insulin-like growth factor 1 receptor) and FAK (focal adhesion kinase) all play imperative roles in the formation and advancement of prostate tumors[6,15,34,123-125]. Extracellular vesicles derived from PCa exhibit significant concentrations of these proteins. Angiogenesis is stimulated by the cross-talk that occurs between Src and IGF-1R[34,126]. Extracellular vesicles rich in Src have been shown to have the ability to stimulate angiogenesis in animal models that have prostate tumors. This is

because Src is overexpressed within plasma extracellular vesicles of mice with prostate tumors. Furthermore, Src and IGF1-R affect angiogenesis by activating VEGF and VEGF-C, respectively[127,128]. This is the case for both of these and focal adhesion kinase, which, if activated, leads to metastasis and angiogenesis[34,129]. Cancer extracellular vesicles that contain TGF-β play critical roles in the formation of stroma that is favorable to the development of tumors. These extracellular vesicles encourage the differentiation of fibroblasts into a myofibroblast-like phenotype, which in turn stimulates angiogenesis and tumor expansion[130-131]. The activation of the TGF-β/SMAD3 cascade is the mechanism by which this impact is produced[132]. PCa cells are responsible for producing extracellular vesicles that have a high level of latent TGF-β expression. This latent TGF-β adheres to the exosome surface via proteoglycans, promoting the activation of SMAD3-dependent signaling cascades[133,134]. Figure 2 shows a graphical representation of pro-tumorigenic characteristics of extracellular vesicles in PCa.

THE ROLE OF EXTRACELLULAR VESICLES IN THE DIAGNOSIS AND THERAPY OF PCa

Utilization of extracellular vesicles in the of PCa diagnosis-recent advances

On the positive side, extracellular vesicles are widely studied for the diagnosis and therapy of PCa[135,136]. The extracellular vesicles derived from the urine, plasma, and semen[40,137,138] have been utilized for the diagnosis of PCa. Several studies demonstrated the utilization of extracellular vesicles in PCa diagnosis[39,139-141]. A recent clinical investigation revealed that PCa patients may be discriminated from both Benign Prostate Hyperplasia (BPH) and healthy subjects by the expression of PSA on plasmatic extracellular vesicles. In a study, it was demonstrated as a new strategy for differentiating not just PCa from healthy persons but also from benign hypertrophy[142]. In another study, it was found that extracellular vesicles from individuals with PCa exhibited overexpression of CA IX levels, which is related to intraluminal pH, as compared to healthy persons, and demonstrated that the PCa extracellular vesicles are acidic in nature and can be used as a biomarker in PCa[143]. Since the literature depicts the use of extracellular vesicles in the diagnosis of PCa, in this review, we will focus on the latest advances in extracellular vesicles for the diagnosis of PCa. Li *et al.* developed a superparamagnetic conjunctions and molecular beacons (SMC-MB) based platform in which it detected and captured the prostate-specific membrane antigen-positive extracellular vesicles, thereby depicting its efficiency in the diagnosis of PCa[144].

In another study, an economical, easy, and non-invasive method was developed for diagnosing PCa in which the extracellular vesicles containing surface proteins and miRNAs from extracellular vesicles were detected at the same time and permitted the analysis of particular miRNAs and surface proteins through one reaction[145]. A nanoplatform for specific and quick detection, which consists of off-on signal responses and reversible conjunction, was developed in which high-affinity particles of Fe_3O_4@SiO_2@TiO_2 were employed for exosome capture and selectivity was improved through the fluorescence response PMSA aptasensor which aid in the tumor exosome detection. The study concluded that this method was fruitful for quick diagnosis[146]. In another study reported, a microfluidic Raman biochip with immunoassay was developed for the separation and analysis of extracellular vesicles. This chip was able to differentiate the samples of PCa patients and normal samples. Furthermore, this system detected the extracellular vesicles in one hour and thus can be explored as the detection test for PCa[147]. In summary, here we discussed some of the recent advances in the utilization of extracellular vesicles for the diagnosis of PCa. To the best of our knowledge, extracellular vesicles have been extensively utilized for the detection of PCa, and various advances such as the Raman chip and others have also been developed and employed for much rapid, non-invasive and sensitive analysis of the samples. Soon, various additional options will be developed to enhance diagnosis using extracellular vesicles.

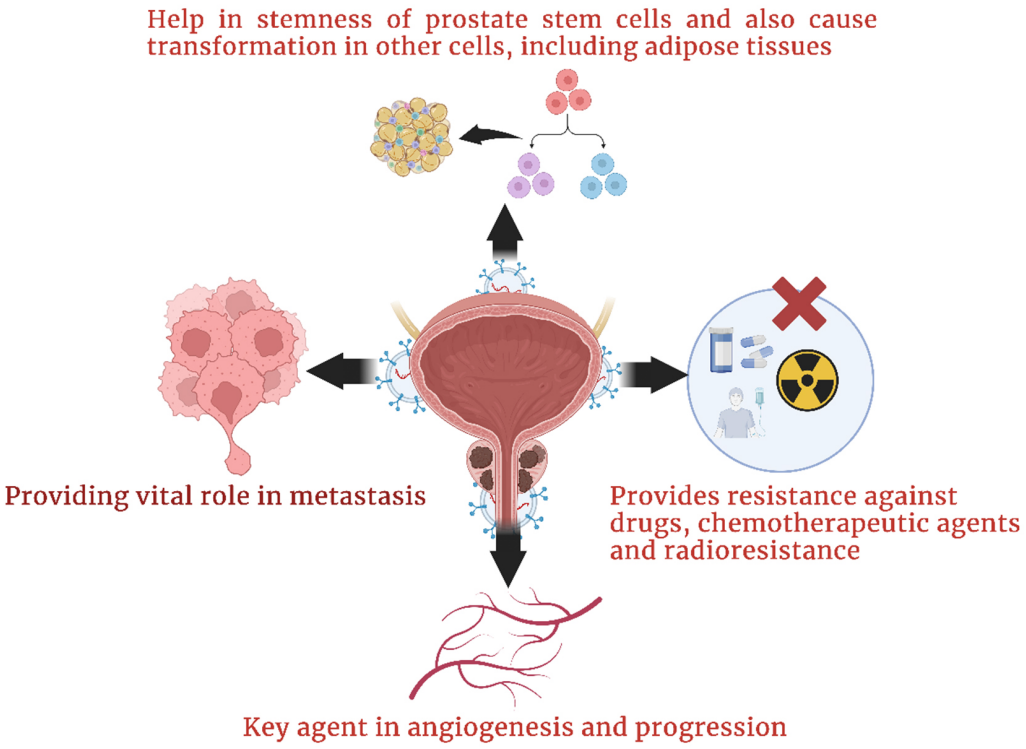

Figure 2. Graphical representation of the pro-tumorigenic characteristics of extracellular vesicles in PCa.

Extracellular vesicles in the PCa therapy

Another such advantage is its application in drug delivery. Owing to their advantages over conventional delivery systems, such as their high ability to overcome biological barriers, enhanced stability, better targeting, unnecessary accumulation in the liver, and low toxicity, extracellular vesicles are widely utilized for the delivery of drugs as well as other therapeutic moieties such as siRNA, phytoconstituents[28,148]. The extracellular vesicles are obtained from various cells, such as tumor cells, immune cells, and mesenchymal stem cells, and are also derived from bovine milk. Of these, the extracellular vesicles derived from mesenchymal stem cells and tumor cells are widely explored for therapeutic purposes[149]. In this section, the role of extracellular vesicles in the delivery of various therapeutic moieties such as the siRNA, chemotherapeutics, and phytoconstituents are discussed [Figure 3].

siRNA delivery through extracellular vesicles

It was shown that due to the underlying mechanisms, extracellular vesicles are found to be the right candidates for the delivery of siRNA[148]. For this reason, extracellular vesicles are utilized for the successful delivery of siRNA for targeting various cancers such as breast cancer[150], colon cancer, gastric cancer, and others, including PCa[151,152]. In the context of PCa, a research study was aimed to determine the efficiency of siRNA in the inhibition of SIRT6 in a PCa model. It was demonstrated that the siRNA via the engineered extracellular vesicles caused the downregulation of the SIRT6 and blocked the metastasis and tumor growth[153]. In another study by Krishn *et al.*, it was demonstrated that delivery of siRNAs targeting ITGB6

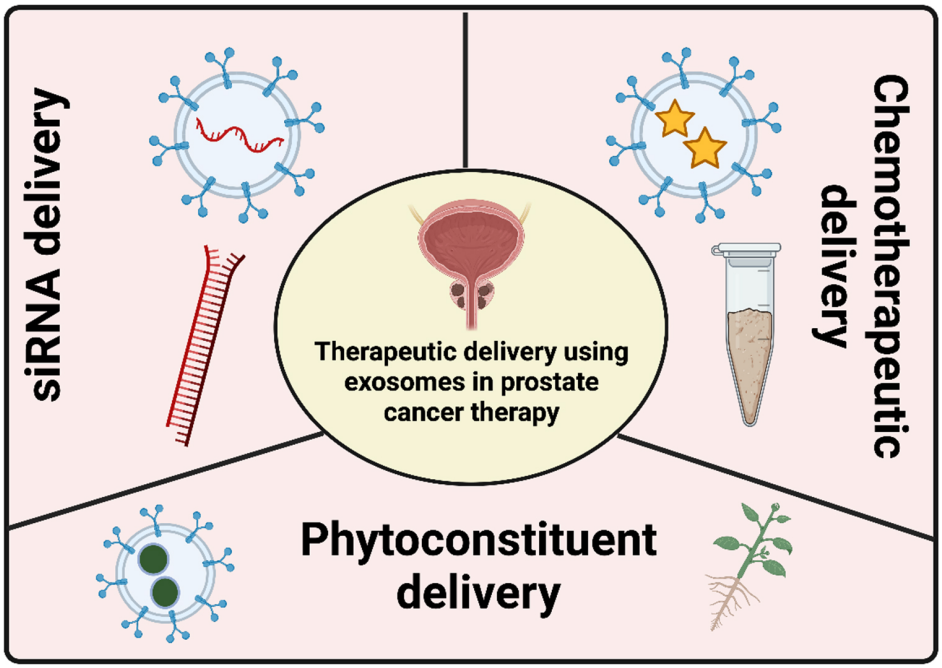

Figure 3. The application of extracellular vesicles in the therapy PCa.

into the PCa cells (PC3) led to the downregulation of β6 subunit expression and also led to cell adhesion and migration of PCa cells on a specific substrate of αVβ6[154]. In an *in vivo* study on mice bearing subcutaneous prostate carcinoma, the extracellular vesicles modified with polyethyleneimine showed strong inhibition of tumor growth, which is due to the significant knockdown of the target gene[155]. Even though siRNA delivery through extracellular vesicles has been researched widely in recent times, in our opinion, the extracellular vesicles can be utilized to a greater extent for the targeted therapy of PCa due to their effectiveness in inhibiting the target genes as well as the advantages it offers.

Drug delivery through extracellular vesicles

The extracellular vesicles are also used to carry drugs to the delivery site [Figure 3]. Followed by its isolation, the drugs are conjugated with them, which can reduce the toxicity and biocompatibility issues[156]. Saari *et al.* isolated the extracellular vesicles from PCa cells (PC-3 and LNCaP) through a process called differential centrifugation. An increased cytotoxic effect was observed when the paclitaxel was loaded into the extracellular vesicles derived from autologous PCa cells. Furthermore, endocytosis was the key pathway involved in the delivery of paclitaxel to the parental cells, thereby demonstrating the effectiveness of extracellular vesicles as carriers for drug delivery[83]. In another study, the urinary-derived exosomal system containing doxorubicin Exo-PMA/Fe-HSA@DOX nano vectors was explored for synergistic chemodynamic/low-dose chemotherapy of PCa. The nanosystem was shown to cause substantial internalization *in vitro* and to suppress the EGFR/AKT/NF-B pathways in cells[157]. Not just drugs, but a combination of both drugs and siRNAs have also been delivered for PCa therapy. For example, folate-conjugated extracellular vesicles (Co-Exo-FA) were developed which are obtained from nano-complex

loaded macrophages. Docetaxel and PLK1 siRNA were loaded in this system. It was shown that the system blocked the PLK1 gene in addition to its effect on tumor growth and reduced toxicity, which demonstrated the synergistic effect of drug and siRNA combination against castrate-resistance PCa[158].

Phytoconstituent delivery through extracellular vesicles

It has been reported that extracellular vesicles aid in the delivery of phytochemicals across biological barriers and have also been used against cancer[159]. However, in PCa, there are very few phytochemicals delivered through the exosome in our knowledge. Overall, extracellular vesicles are utilized to transport various molecules to PCa patients, and still, a lot of molecules such as various siRNAs, drugs, and phytochemicals can be loaded into extracellular vesicles for the targeted inhibition of various genes associated with PCa, tumor growth and to target the various steps involving in the emergence of PCa. In our opinion, this area remains much unexplored.

CONCLUSION AND FUTURE PERSPECTIVE

Extracellular vesicles show a significant role when it comes to the entire scenario involving PCa. Once considered garbage bags of cells, extracellular vesicles are now being researched for their multifarious roles in cancer development. In the case of PCa, they have shown a dual persona: a negative aspect enables extracellular vesicles to contribute to proliferation and metastases while conferring resistance against the majority of chemotherapeutic agents and therapies, including radiation therapy. However, they also demonstrate a beneficial side, serving as diagnostic biomarkers and delivery vesicles for a vast array of agents, including nucleic acids, diagnostic agents, pharmaceuticals, and many more. This review aims to comprehensively cover both aspects of extracellular vesicles in PCa, encompassing their negative roles, such as involvement in angiogenesis, metastasis, proliferation, stemness, and resistance to multiple agents, alongside their roles as diagnostic agents and delivery agents for various pharmaceuticals.

There is a promising future for extracellular vesicles in treating PCa. While extracellular vesicles have shown tremendous progress as delivery vesicles for multiple agents, there is a limited number of studies focused on the delivery of phytochemicals in PCa using extracellular vesicles. Additionally, further studies can be conducted to explore how their role in drug resistance can be utilized positively while delivering antineoplastic agents in PCa. PCa has been a major threat in the male population, but future studies and the utilization of extracellular vesicles are expected to play a significant role in its treatment and mitigating its effects worldwide.

DECLARATIONS

Author contributions

Writing original draft: Jain DP
Conceptualization, writing original draft: Dinakar YH
Writing, reviewing, and editing: Kumar H, Jain R
Conceptualization, writing, reviewing, editing, and supervision: Jain V

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Cancer Drug Resistance

Review

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The clinical challenges of homologous recombination proficiency in ovarian cancer: from intrinsic resistance to new treatment opportunities

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Abstract

Ovarian cancer is the most lethal gynecologic cancer. Optimal cytoreductive surgery followed by platinum-based chemotherapy with or without bevacizumab is the conventional therapeutic strategy. Since 2016, the pharmacological treatment of epithelial ovarian cancer has significantly changed following the introduction of the poly (ADP-ribose) polymerase inhibitors (PARPi). *BRCA1/2* mutations and homologous recombination deficiency (HRD) have been established as predictive biomarkers of the benefit from platinum-based chemotherapy and PARPi. While in the absence of HRD (the so-called homologous recombination proficiency, HRp), patients derive minimal benefit from PARPi, the use of the antiangiogenic agent bevacizumab in first line did not result in different efficacy according to the presence of homologous recombination repair (HRR) genes mutations. No clinical trials have currently compared PARPi and bevacizumab as maintenance therapy in the HRp population. Different strategies are under investigation to overcome primary and acquired resistance to PARPi and to increase the sensitivity of HRp tumors to these agents. These tumors are characterized by frequent amplifications of Cyclin E and MYC, resulting in high replication stress. Different agents targeting DNA replication stress, such as ATR, WEE1 and CHK1 inhibitors, are currently being explored in preclinical models and clinical trials and have shown promising preliminary signs of activity. In this review, we will summarize the available evidence on the activity of PARPi in HRp tumors and the ongoing research to develop new treatment options in this hard-to-treat population.

Keywords: PARP inhibitors, homologous recombination proficiency, ovarian cancer, PARP inhibitor resistance, replication stress

INTRODUCTION

Ovarian cancer (OC) is the deadliest gynecological tumor and the 13th leading cause of cancer mortality in the United States. In 2021, 21,410 new cases of OC were estimated, with 13,770 deaths due to this disease[1]. Among malignant ovarian tumors, epithelial ovarian cancer (EOC) constitutes the majority, with high-grade serous ovarian cancer (HGSOC) being the most common histological subtype[2]. The diagnosis of HGSOC commonly occurs at an advanced stage due to the consequence of non-specific symptoms and a lack of effective screening strategies[3]. The standard treatment combines optimal debulking surgery and platinum-based chemotherapy. Nevertheless, the majority of patients develop recurrence and the efficacy of subsequent lines of treatment decreases over time[4]. Maintenance treatment was introduced to delay disease progression after first-line treatment. The antiangiogenic agent bevacizumab has been the first targeted agent to receive authority approval in first-line treatment of International Federation of Gynecology and Obstetrics (FIGO) stage III and IV EOC[5,6]. Subsequently, the development of poly (ADP-ribose) polymerase inhibitors (PARPi) has represented a paradigm change in the treatment of EOC and they are now incorporated into the standard of care treatment[7].

PARPi IN OVARIAN CANCER

Poly (ADP-ribose) polymerases (PARPs) are responsible for the detection of single-strand DNA breaks (SSBs) and the recruitment of DNA repair factors at the site of DNA damage[8]. Following PARP inhibition, SSBs are not properly repaired and PARP is trapped and the replication fork stalled with the subsequent occurrence of DNA double-strand breaks (DSBs)[8,9]. These DNA damages activate, leading to an error-free DNA damage repair.

Approximately 15% of patients with EOC harbor a germline mutation in *BRCA1/2* (*gBRCA*)[10]. *BRCA1* and *BRCA2* are tumor suppressor genes involved in multiple cellular pathways, such as transcription, cell cycle regulation, and maintenance of genome integrity[11]. Specifically, they are involved in DNA DSB repair and are essential for the HRR pathway.

PARPi are particularly active in cancer cells with alterations in HRR, such as *BRCA1* or *BRCA2* pathogenic mutations, through the well-known mechanism of "synthetic lethality"[12,13]. With the loss of HRR function, cells repair DSBs via the non-homologous end joining (NHEJ), resulting in genomic instability and cell death.

PARPi have been studied in high-grade EOC in different phase II and III clinical trials as maintenance and treatment strategies, leading to approvals by health authorities for different indications[14-23].

HOMOLOGOUS RECOMBINATION DEFICIENCY

The most disruptive form of DNA damage is the DNA DSBs, which are repaired by NHEJ and homologous recombination[24]. When DNA damage occurs, different enzymes are activated, including the MRE11-RAD50-NBS1 complex (MRN), the DNA damage kinase ataxia-telangiectasia mutated (ATM), and the ataxia telangiectasia and Rad3-related (ATR). Subsequently, DNA repair is activated by *CHEK2*, *BRCA1*, *BRCA2*, and *RAD51*. Other important effectors of the HRR pathway are *PALB2* and *BRIP1*[11]. A defect in the HRR process determines a condition called homologous recombination deficiency (HRD) that occurs as a consequence of germline or somatic mutations, or epigenetic modifications of different genes involved in this pathway.

Almost 50% of high-grade EOC are characterized by HRD, with 12%-15% and 5%-7% harboring germline or somatic mutations in *BRCA1/2*, respectively[25-27]. However, HRD also occurs due to germline or somatic mutations or epigenetic events in other HRR genes, and not all mechanisms underscoring HRD have been characterized yet[27,28].

HRD assays and their limitations

Different types of assays, referred to as "HRD tests", are available to select patients more likely to benefit from PARPi[28]. HRD can be measured using different strategies, which can be categorized into four main groups and each of them has its own limitations[28,29].

Germline or somatic mutations in HRR genes

Next-generation sequencing (NGS) of DNA extracted from peripheral blood or saliva is used to detect germline pathogenic variants of genes involved in HRR. In patients without a g*BRCA* mutation, a somatic test on formalin-fixed paraffin-embedded (FFPE) tissue is recommended, given ~5%-7% of EOC harbor a somatic mutation in *BRCA1/2*[27,30,31]. In addition to *BRCA1/2* mutations, other causes of HRD include promoter methylation of *BRCA1* (10%) or *RAD51C* (3%) or mutations in other genes such as ATM or ATR (~2%), CDK12 (~3%), *BRIP1*, *RAD51C*, *RAD51D*, *PALB2*, and BARD1 (~5%)[30]. Different studies demonstrated similar efficacy of PARPi in patients with somatic compared to germline *BRCA* mutations[20,32,33]. Mutations in HRR genes other than *BRCA* are rare, with only limited data available on the benefit of PARPi in this setting. A retrospective analysis of the PAOLA-1 trial demonstrated that the presence of non-*BRCA* HRR gene mutations, contrary to HRD positive status, was not predictive of progression-free survival (PFS) benefit[34]. The exploratory analysis of the ARIEL2 trial showed that the presence of loss of heterozygosity (LOH) in *BRCA* wild-type tumors was a more sensitive biomarker of response compared to other HRR gene mutations or *BRCA1* and *RAD51C* methylation[23,33]. An important challenge in the identification of germline and somatic mutations is the definition of the functional and clinical meaning of variants of uncertain significance (VUS) and their correlation with benefit from PARPi[35]. Despite the work of the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium to reclassify many VUS as benign or malignant, the absolute number of individuals receiving an inconclusive *BRCA1/2* test result is still significant[36].

Genomic "scars" and mutational signatures

HRD causes genomic instability with a progressive accumulation of genomic aberrations that result in "scars" that can be measured with specific assays. These assays do not look for the cause of HRD, but to the consequences of having a defective HRR pathway. Several investigations have been conducted using single nucleotide polymorphism arrays to define a signature of genomic instability associated with *BRCA* mutations. The three major types of genomic "scars" associated with HRD are LOH, telomeric allelic Imbalance (TAI) and large-scale state transitions (LST)[37-39]. Two commercially available genomic instability assays have been developed: the myChoice® CDx from Myriad Genetics and the FoundationFocus™ $CDx_{BRCA\ LOH}$ by Foundation Medicine. The myChoice® CDx assay evaluates tumor *BRCA1/2* mutation (t*BRCA*), LOH, TAI and LST with a genomic instability score (GIS) cut off at 42[15,16]. The FoundationFocus™ assay evaluates t*BRCA* and genomic LOH, with an initial cut-off for LOH positivity established at 14%[26]. This cut-off was initially prospectively evaluated in the ARIEL2 trial[23], but subsequently changed to 16% according to the ARIEL3 trial results, which corresponds to the cut-off currently used in the available assay[22].

One of the major limitations of these genomic instability assays is the inability to define the current HRD status. Genomic scars can persist even if tumor cells have restored HRR through mechanisms, such as the

occurrence of reversion mutations in *BRCA* and other HRR-related genes[40-42]. Notably, different cut-offs have been employed to define the presence of HRD in the different clinical trials evaluating the role of PARPi in EOC, and direct comparisons among the two approved assays are not available. Other limitations of the currently commercially available HRD tests are the limited reproducibility, their availability and cost. Several research groups are validating academic tests with the aim of overcoming such limitations and preliminary results have been recently presented[43,44].

Mutational signatures can also be used to identify the presence of HRD. This method is based on the assumption that several mutational processes, such as smoking, carcinogens, ultraviolet radiation or defects in DNA repair mechanisms, cause somatic mutations and each of them generates a specific mutational signature[45]. Signature 3 is the one associated with *BRCA* mutation and *BRCA1* promoter methylation in different solid tumors, including ovarian cancer[45-47]. Hillman *et al.* conducted a retrospective whole genome sequencing (WGS) of HGSOC samples and demonstrated that the presence of signature 3 correlates with better prognosis and response to platinum agents[48]. However, the most common genomic testing platform used in clinical practice are targeted sequencing panels with a number of identifiable mutations too small for the identification of a specific signature. To overcome this limitation, Gulhan *et al.* have developed a bioinformatics algorithm (Signature Multivariate Analysis-SigMA), which can identify the mutational signature correlated with HRD using genetic panels[49]. In their analyses, cancer cell lines with signature 3 showed greater sensitivity to PARPi, irrespective of the *BRCA* mutational status, not only in breast and ovarian cancers but also in other tumor types[49].

Similar to other genomic "scars" assays, the identification of a mutational signature linked to defects in DNA repair pathways also remains a historical representation of HRD and does not reflect the actual presence of the mechanism of resistance to PARPi. Another major limitation is the need for fresh frozen material to perform these assays. Despite being a promising approach, it lacks clinical validation[28].

Functional assays

Functional assays are a dynamic assessment of the HRR status[28]. In the presence of DNA DSBs, RAD51 forms molecular complexes (foci) at the DNA damage site, which are visible by immunofluorescence microscopy. It has been shown that cell lines with HRD are not able to form RAD51 foci when DNA damage occurs and this represents a functional reflection of a defect in the homologous recombination pathway[50-52]. Blanc-Durand *et al.* evaluated a RAD51 functional assay in tumor samples from patients enrolled in the randomized neoadjuvant CHIVA trial assessing platinum-based chemotherapy +/- nintedanib[53,54]. They demonstrated that RAD51-deficient ovarian tumors had better overall response rates (ORR) to neoadjuvant chemotherapy and median PFS. Notably, among *BRCA1/2* mutated EOC, patients with RAD51-proficient tumors had a poorer response to chemotherapy[53,54]. RAD51 measurement as a surrogate for HRD has several limitations. Not all mechanisms underlying PARPi sensitivity involve a deficiency in RAD51. When PARPi sensitivity is driven by ATM or MRN complex alterations, RAD51 assay is not able to detect it because these mechanisms preserve the formation of RAD51 foci[50]. Similarly, if the resistance to PARPi is due to a RAD51-independent mechanism, such as mutations in PARP1, it is not identified by a RAD51 assay[55]. Additionally, the assay relies on counting and quantifying many foci, which may result in substantial inter-observer variability[56]. The available data on RAD51 functional assays are retrospective and performed on a limited number of samples[56]. A prospective trial that stratifies patients based on the presence of RAD51 foci is needed to validate its clinical utility.

PARPi IN HRp TUMORS

EOC associated with *gBRCA* mutations has different clinical features, such as earlier age of onset, longer survival, higher rate of visceral metastasis (e.g., liver, spleen), higher response rates to platinum and non-platinum chemotherapy (e.g., pegylated liposomal doxorubicin), and increased sensitivity to PARPi[57-60]. It has become evident that many HGSOCs share the same biological and clinical features even when a germline or somatic *BRCA* mutation is not detected, a condition called "BRCAness"[27]. In contrast, patients with HRp tumors have worse PFS and OS compared to patients with HRD tumors[61,62]. A retrospective analysis of 1,271 patients with EOC showed that patients with HRp tumors have an older median age at diagnosis, have more commonly non-serous histology, require a higher number of neoadjuvant chemotherapy cycles to be considered for interval debulking surgery, and are less sensitive to platinum-based chemotherapy[62,63].

Homologous recombination-proficient EOC also harbors different genomic features. Amplification of cyclin E1 (CCNE1) is one of the best characterized genomic alterations linked to resistance to platinum-based chemotherapy and is mutually exclusive with *BRCA1/2* dysfunction[64,65]. CCNE1 encodes the cell-cycle regulator cyclin E1, which is required for the cell-cycle progression from G1 to S through the p21-p27-cyclin E-CDK2 pathway. CCNE1 amplification raises cyclin E levels, leading to abrogation of the G1-S cell cycle checkpoint and reduction of the G1 phase[66]. This results in high replicative stress (RS) due to aberrant firing of the replication origin[66]. MYC amplification is also found in HRp cancer and correlates with increased RS. MYC-amplified cancer cells are characterized by inadequate reduction/oxidation balance with accumulation of reactive oxygen species, accelerated entry in S phase and impairment in the expression of genes implicated in purine and pyrimidine biosynthesis and in the regulation of the HR pathway[66,67].

While recent clinical trials have clearly proved the activity of PARPi in the presence of *BRCA* mutation and/or HRD, their role in the HRp population is less understood.

In the first-line setting, three trials have investigated the role of maintenance treatment with a PARPi following a complete or partial response (CR/PR) to chemotherapy [Table 1]. The PRIMA trial is a phase 3 randomized trial of niraparib *vs.* placebo as maintenance treatment in patients with high-risk stage III (residual disease after debulking surgery, inoperable stage III and patients who have received neoadjuvant chemotherapy) and stage IV high-grade EOC. Although an improvement in median PFS in the overall population regardless of HRD status [defined as *BRCA* mutation or/and a GIS score ≥ 42 (myChoice® assay)] was obtained with niraparib, in the HRp population, the benefit was smaller[2]. The phase III PAOLA-1 trial investigated the addition of olaparib to bevacizumab as maintenance treatment in newly diagnosed, stage III-IV high-grade EOC after response to first-line chemotherapy[15]. This study demonstrated a statistically and clinically significant improvement in PFS and overall survival (OS) in the overall population, in the HRD-positive/t*BRCA* and HRD-positive/*BRCA*wt groups, but with no difference in the HRp population[15,68]. Recently, the phase 3 ATHENA-MONO trial has confirmed the activity of rucaparib regardless of the HRD status, measured as LOH using the FoundationOne CDx NGS assay[69].

The combination of veliparib with chemotherapy containing platinum compound in stage III/IV EOC was evaluated in the phase 3 VELIA trial[70]. Patients were randomized in three different arms: chemotherapy plus placebo followed by placebo maintenance, chemotherapy plus veliparib followed by placebo maintenance, or chemotherapy plus veliparib followed by veliparib maintenance. Veliparib, administered concomitantly with chemotherapy and as maintenance treatment, improved patients' outcomes (PFS from the start of first-line chemotherapy) in the three prespecified subgroups: *BRCA* mutated, HRD positive and the overall population. The HRD population includes patients with *BRCA* mutated tumors and/or a GIS

Table 1. Results of the main randomized phase 2 and 3 clinical trials investigating PARPi in EOC, including HRp population

Trial	PRIMA	PAOLA-1	ATHENA-MONO	VELIA	NOVA	ARIEL3	AVANOVA
Treatment	Niraparib *vs.* placebo	Olaparib + bev *vs.* placebo + bev	Rucaparib *vs.* placebo	Veliparib + cht → veliparib *vs.* cht + placebo → placebo[&]	Niraparib *vs.* placebo	Rucaparib *vs.* placebo	Niraparib + bev *vs.* niraparib + placebo
Setting	First line maintenance	First line maintenance	First line maintenance	First line with chemo and maintenance	Platinum-sensitive recurrence maintenance	Platinum-sensitive recurrence maintenance	Platinum sensitive recurrence, ≤ 1 prior non-platinum lines
PFS ITT (months)	13.8 *vs.* 8.2 (HR 0.62)	22.1 *vs.* 16.6 (HR 0.59)	20.2 *vs.* 9.2 (HR 0.52)	23.5 *vs.* 17.3 (HR 0.68)	NA	10.8 *vs.* 5.4 (HR 0.36)	11.9 *vs.* 5.5 (HR 0.35)
PFS *BRCA* mut (months)	22.1 *vs.* 10.9 (HR 0.40)	37.2 *vs.* 21.7 (HR 0.31)	NR *vs.* 14.7 (0.40)	34.7 *vs.* 22 (HR 0.44)	21 *vs.* 5.5 (HR 0.27)	16.6 *vs.* 5.4 (HR 0.23)	14.4 *vs.* 9 (HR: 0.49)
PFS HRDpos (months)	21.9 *vs.* 10.4 (HR 0.43)	37.2 *vs.* 17.7 (HR 0.33)	28.7 *vs.* 11.3 (HR 0.47)	31.9 *vs.* 20.5 (HR 0.57)	12.9 *vs.* 3.8 (HR 0.38)	13.6 *vs.* 5.4 (HR 0.32)	11.9 *vs.* 6.1 (HR 0.38)
PFS HRDpos/ *BRCA*wt (months)	19.6 *vs.* 8.2 (HR 0.50)	28.1 *vs.* 16.6 (HR 0.43)	20.3 *vs.* 9.2 (HR 0.58)	NA	9.3 *vs.* 3.7 (HR 0.38)	[*]9.7 *vs.* 5.4 (HR 0.44)	11.9 *vs.* 4.1 (HR 0.19)
PFS HRp (months)	8.1 *vs.* 5.4 (HR 0.68)	16.9 *vs.* 16 (HR 0.92)	12.1 *vs.* 9.1 (HR 0.65)	15 *vs.* 11.5 (HR 0.81)	6.9 *vs.* 3.8 (HR 0.58)	[**]6.7 *vs.* 5.4 (HR 0.58)	11.3 *vs.* 4.2 (HR 0.40)
HRD test	Myriad MyChoice®	Myriad myChoice HRD Plus®	FoundationOne CDx	Myriad myChoice HRD CDx®[§]	Myriad MyChoice HRD®	Foundation Medicine T5 NGS	Myriad MyChoice HRD®
Role of HRD score	Stratification factor	Prespecified subgroup analysis	Stratification factor	Prespecified subgroup analysis	Prespecified subgroup analysis	Stratification factor	Stratification factor

[*]BRCAwt, high LOH; [**]BRCAwt, low LOH; [&]Data of the veliparib combination only arm are not reported; [§]Initially the cut off for HRD positivity was ≥ 33, subsequently revised to ≥ 42. bev: Bevacizumab; BRCAmut: BRCA mutated; BRCAwt: BRCA wild type; cht: chemotherapy; EOC: epithelial ovarian cancer; HR: hazard ratio; HRD: homologous recombination deficiency; HRp: homologous recombination proficient; ITT: intention-to-treat; LOH: loss of heterozygosity; NA: not available; NR: not reach; PARPi: poly (ADP-ribose) polymerase inhibitors; PFS: progression-free survival; *vs.*: versus.

score ≥ 42 (myChoice® assay). In the HRp subgroup, the effectiveness of the standard of care treatment was not enhanced by the addition of velaparib[70].

In the platinum-sensitive recurrent setting, niraparib and rucaparib have shown effectiveness in the different biomarker subgroups. However, as described for the first line setting, there is a different magnitude of benefit, with higher efficacy observed in the *BRCA* mutated population followed by the HRD non-*BRCA* mutated and the HRp subgroups. This was clearly observed in the phase 3 NOVA trial, where the role of niraparib versus placebo as maintenance therapy in platinum-sensitive recurrent high-grade EOC was assessed[20]. HRD positivity was defined when the GIS by myChoice® score was ≥ 42 or a *BRCA1/2* mutation was present. To be noted, a potential detrimental effect on OS has been reported, and final data are awaited[20,71].

In the same setting, the ARIEL3 trial evaluated rucaparib as a maintenance treatment, showing an improvement in median PFS in the intention-to-treat population in HRD population, defined as high-LOH (LOH score ≥ 16%) or *BRCA* mutated. As expected, the PFS benefit was greater in patients with LOH high-*BRCAwt* compared to LOH low patients[22].

PARPi have also been investigated as a line of treatment in the ARIEL2[23] and QUADRA trials[21]. QUADRA was a single-arm phase II study where niraparib was administered as monotherapy in patients with recurrent HGSOC after the failure of three or more previous regimens. HRD tumors included *BRCA* mutated and/or myChoice® HRD score ≥ 42. The ORR was 28%, with a median duration of response similar in the different subgroups of patients. Median OS was 26.0 months (95%CI, 18.1-not estimable), 19.0 months (14.5-24.6), and 15.5 months (11.6-19.0) in the *BRCA* mutated, HRD-positive, and HRp populations[21]. The phase 2 ARIEL2 trial assessed the role of rucaparib as treatment. Part 1 of the trial included patients with platinum-sensitive high-grade EOC treated with one or more prior chemotherapy regimens. The analysis has been performed according to three prespecified subgroups: *BRCA* mutant, LOH high and *BRCAwt* (cut off for LOH high 14%), or LOH low. Median PFS with rucaparib treatment was 12.8 (95%CI 9.0 to 14.7), 5.7 months (5.3 to 7.6) and 5.2 months (3.6 to 5.5) in the *BRCA* mutant, LOH high and LOH low patients, respectively. Part 2 of the trial enrolled patients with any platinum-free interval disease who had completed 3 to 4 prior chemotherapies. Patients with *BRCA* mutated tumors had a median PFS of 7.8 months and an ORR of 45.7% (95%CI, 37.2 to 54.3). In contrast, in the LOH-high and LOH-low groups, median PFS was 4.3 and 4.0 months and ORR was 16.7% (95%CI, 11.2 to 23.5) and 7.7% (95%CI, 4.2 to 12.9), respectively[23].

The AVANOVA2 is a phase 2 trial that compared niraparib versus the combination of niraparib plus bevacizumab in patients with platinum-sensitive recurrent ovarian cancer. In the prespecified subgroup analysis, a longer PFS was observed with the combination regardless of the HRD status[72].

Results of the main phase 2 and 3 trials of PARPi in EOC that have included HRp patients are summarized in Table 1 and Figure 1A and B.

MAINTENANCE TREATMENT IN HRp TUMORS: PARPi OR ANTIANGIOGENIC AGENTS

The antiangiogenic agent bevacizumab has demonstrated an improvement in PFS when used with chemotherapy and as maintenance in first-line settings, but also in platinum-sensitive recurrent disease[5,6,73,74]. The GOG-218 is a 3-arm phase 3 trial assessing the efficacy of bevacizumab added to first-line chemotherapy in patients with stage III and residual disease after surgery and stage IV EOC. Patients were randomized to receive six cycles of carboplatin, paclitaxel and placebo followed by placebo (arm 1), or the same chemotherapy with the addition of bevacizumab (15 mg/kg) followed by placebo maintenance (arm 2) or carboplatin and paclitaxel with bevacizumab and bevacizumab maintenance up to 22 total administrations (arm 3). Treatment with bevacizumab concurrent plus maintenance reduced the risk of disease progression by 28% (median PFS 14.1 *vs.* 10.3 months; HR = 0.717; 95%CI, 0.625 to 0.824)[5]. The difference in OS was noted only in the subgroup of patients with stage IV disease. A retrospective analysis evaluated the impact of HRR gene mutations (such as *ATM, ATR, BARD1, BRCA1, BRCA2, BRIP1, CHEK2, PALB2, RAD51C, RAD51D, and others*) on OS, platinum and bevacizumab sensitivity (comparing arm1 and 3)[75]. This analysis demonstrated that mutations in HRR might be correlated with a better PFS and OS in patients with EOC, but bevacizumab did not result in a different benefit according to the mutational status[75].

A small retrospective study analyzed the role of CCNE overexpression in predicting the benefit of bevacizumab in 57 patients with platinum-sensitive recurrence of OC. 45.6% of the patients presented CCNE1 overexpression and 15 (62.5%) were treated with chemotherapy and bevacizumab and 11 (33.3%) received chemotherapy alone. Among patients with CCNE1 overexpression, the ORR was 100% and 50% in the group treated with or without bevacizumab, respectively. The PFS was higher in patients with CCNE1 overexpression who received bevacizumab (16.3 *vs.* 7.1 months, $P = 0.010$)[76].

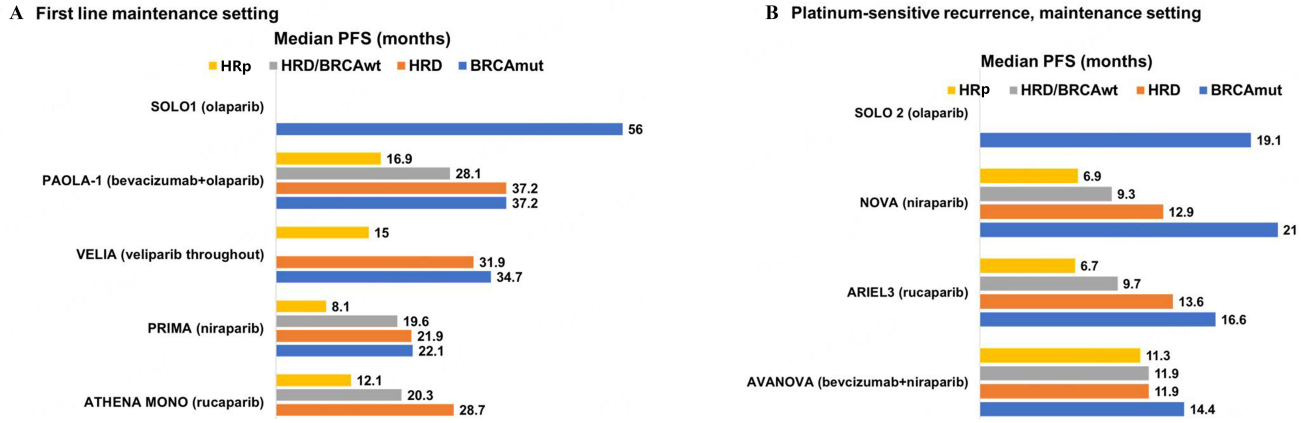

Figure 1. Indirect comparison of PFS with PARPi maintenance according to *BRCA* or HRD/LOH status in the main randomized phase 3 trials. In VELIA trial, PFS was calculated from the start of chemotherapy. ATHENA MONO: PFS in BRCA mutated population is not reached. BRCAmut: BRCA mutated; BRCAwt: BRCA wild type; HRD: homologous recombination deficiency; HRp: homologous recombination proficiency; LOH: loss of heterozygosity; PARPi: poly (ADP-ribose) polymerase inhibitors; PFS: progression-free survival; VELIA: PSF in HRD/BRCAwt not reported.

To date, no randomized trials comparing PARPi plus bevacizumab *vs.* PARPi alone, or PARPi *vs.* bevacizumab are available. Hettle *et al.* performed a population-adjusted indirect treatment comparison among the high-risk population of the PAOLA-1 trial matched to the PRIMA cohort[77]. The authors compared the relative efficacy of olaparib plus bevacizumab (group 1), bevacizumab alone (group 2), niraparib (group 3) and placebo (group 4). Median PFS in the biomarker unselected population was 21.4 months (95%CI, 19.2 to 22.1) in group 1, 16.0 months (95%CI, 14.3 to 17.7) for patients in group 2, 13.8 months (95%CI, 11.5 to 15.3) for patients in group 3 and 8.1 months (95%CI, 7.31 to 8.51) for patients in the placebo arm. In the HRD population, the greater advantage was achieved with the association of PARPi and bevacizumab than from PARPi alone or bevacizumab alone, with a median PFS of 36.0 months [95%CI, 23.2-not available (NA)] for patients in the olaparib plus bevacizumab arm, 17.6 months (95%CI, 14.7 to 19.6) for patients in the placebo plus bevacizumab arm, 22.0 months (95%CI, 19.3 to NA) for patients in the niraparib arm and 10.5 months (95%CI, 8.05 to 12.1) for patients in the placebo arm. Unfortunately, because of the lack of baseline data for the HRp subgroup of the PRIMA trial, it was not possible to repeat the same comparative analysis in the HRp population[77].

The MITO-MANGO16 trial explored the effect of adding bevacizumab to chemotherapy in patients previously treated with the same antiangiogenic agent and experiencing the first platinum-sensitive recurrence. Bevacizumab beyond progression resulted in a better PFS 11.8 *vs.* 8.8 months (HR 0.51; 95%CI, 0.41 to 0.65). In a non-preplanned subgroup analysis, PFS was not improved in *BRCA* mutated patients[78].

In the GOG-218 and MITO-MANGO16 trials, HRD tests have not been performed, and thus, an indirect comparison with the HRp populations is not possible. Notably, it is important to highlight that in the PARPi trials, the randomization occurred after the end of the chemotherapy and patients without at least a partial response were excluded, while in the bevacizumab trials, the randomization occurred at the time of initiation of first-line treatment. Thus, the comparison of the magnitude of PFS needs to account for this difference[5,78].

The PAOLA-1 trial failed to show a benefit of adding olaparib to bevacizumab in the HRp subgroup (HR 0.92; 95%CI, 0.72 to 1.17)[15]. In the AVANOVA trial, the combination of niraparib and bevacizumab showed a statistically significant improvement in PFS, even in the HRp population (HR 0.40; 95%CI, 0.19 to 0.85)[72]. The main difference between these two studies is the control arm, that is bevacizumab in the

PAOLA-1 trial and niraparib in the AVANOVA. Although a direct comparison among these trials is not feasible, these results raise the question if the HRp population could gain a higher benefit from the treatment with bevacizumab than a PARPi.

The ongoing MITO 25 trial (NCT03462212) might help in answering this question. This is a phase 1/2 trial evaluating the efficacy of carboplatin-paclitaxel and rucaparib maintenance versus carboplatin-paclitaxel-bevacizumab and bevacizumab plus rucaparib maintenance in HRD-positive HGSOC and of carboplatin-paclitaxel-bevacizumab and bevacizumab maintenance versus carboplatin-paclitaxel and rucaparib maintenance in HRp patients.

INCREASING THE EFFICACY OF PARPi IN HRp TUMORS

As previously described, the efficacy of PARPi is significantly different among HRD and HRp tumors. Thus, there is an unmet need to identify new treatment strategies to increase the activity of DNA-damaging agents in this patient population. Preclinical studies have shown that different pathways are involved in the regulation of the DDR[79] [Figure 2]. The phosphoinositide 3-kinase (PI3K) and MEK pathways are involved in recognition of DNA damage and the promotion of homologous recombination, while the cyclin-dependent kinases (CDK) are involved in the control of the cell cycle progression and act in coordination with the DDR pathways[80-83]. Transcriptional and epigenetic regulation has a critical role in the downregulation of HRR genes, particularly due to the close link between the DDR pathway and chromatin remodeling by histone modifications[79]. Targeting these pathways has the potential to pharmacologically induce an HRD phenotype and might represent a powerful strategy to sensitize HRp EOC to PARPi. Several compounds have been evaluated as potential inducers of HRD and preliminary preclinical and clinical results are available [Table 2].

Transcriptional regulators

Histone deacetylases (HDACs) have a key role in gene transcription, DNA replication and repair. Preclinical research has demonstrated that HDAC inhibitors reduce DNA repair by inhibiting HR genes, which, in turn, creates an HRD-like phenotype. This, along with a perturbed replication fork progression, leads to DNA DSBs, irreversible DNA damage, and finally cell death[84]. Gupta *et al.* studied the effects of olaparib and the HDAC inhibitor entinostatin in HRp ovarian cancer xenograft models, demonstrating that the combination reduces the peritoneal spread and prolongs survival[85]. A phase I/II study is ongoing assessing the safety and efficacy of olaparib combined with entinostat in patients with HRp ovarian cancer (NCT03924245).

The bromodomain and extraterminal (BET) protein BRD4 supports gene transcription and is implicated in the expression of proteins that regulate the cell cycle and the DDR. *BRCA* wild-type ovarian cancer cells treated with the BET inhibitor (BETi) JQ1 exhibit a downregulation of WEE1 and the DNA factor TOPBP1[86,87]. Moreover, the BETi INCB054329 directly represses the transcription of *BRCA1* and *RAD51* in cancer cells[88]. The association of olaparib and JQ1 suppresses the growth of HRp EOC xenografts, while there was no significant effect of either BETi or olaparib if used as a single agent[88]. More information on the efficacy of these combinations will become available from the phase II trial of the BETi, ZEN003694, combined with the PARPi, talazoparib, in patients with recurrent ovarian cancer progressed to prior PARPi (NCT05071937).

PI3K and MEK inhibitors

Preclinical studies have shown that treatment with a phosphatidylinositol-3 kinases inhibitor (PI3Ki) decreases *BRCA1* expression and induces an increase of γ-H2AX, a marker of DNA damage[80]. *BRCA1/2*

Table 2. Ongoing clinical trials of PARPi in combination with new agents in EOC

Class of drugs	Phase	Combination	Population	N
HDACi	I/II	Entinostat + olaparib	Platinum-refractory or resistant, HRp EOC	NCT03924245
	I	Belinostat + talazoparib	mBC, mCRPC, EOC progressed to at least one line of chemotherapy	NCT04703920
BETi	II	ZEN003694 + talazoparib	EOC PARPi resistant and platinum-sensitive (PFI > 6 months)	NCT05071937
PI3Ki	I	BKM120 + Olaparib or BYL719 and olaparib	TNBC and EOC, progressed after at least one prior platinum-based chemotherapy	NCT01623349
	I/II	CYH33 + olaparib	Solid tumors with any DDR gene or PIK3CA mutation, including PARPi and platinum-resistant EOC	NCT04586335
	Ib	Copanlisib + niraparib	Platinum-resistant EOC and *BRCA* mutated PARPi resistant EOC	NCT03586661
MEKi	I/II	Selumetinib + olaparib	Solid tumors with RAS pathway alterations and PARPi resistance EOC	NCT03162627
ATRi	I/II	BAY1895344 + niraparib	Part A: DDR deficiency solid tumor. Part B: platinum-resistant/refractory or PARPi-resistant EOC	NCT04267939
	I	M4344 + niraparib	PARPi resistant EOC	NCT04149145
	II	AZD6738 + olaparib	Recurrent EOC	NCT03462342
WEE1i	II	Adavosertib +olaparib	PARPi resistant EOC	NCT03579316
CDK12i	I	Dinaciclib + veliparib	Solid tumors	NCT01434316

ATRi: ATR inhibitors; BETi: BET inhibitor; CDK: cyclin-dependent kinases; EOC: epithelial ovarian cancer; HDACi: histone deacetylases inhibitor; HRp: homologous recombination proficient; mBC: metastatic breast cancer; mCRPC: metastatic castration-resistant prostate cancer; PARPi: poly (ADP-ribose) polymerase inhibitors; PFI: platinum-free interval; PI3Ki: phosphatidylinositol-3 kinases inhibitor; TNBC: triple-negative breast cancer; WEE1i: WEE1 inhibitors.

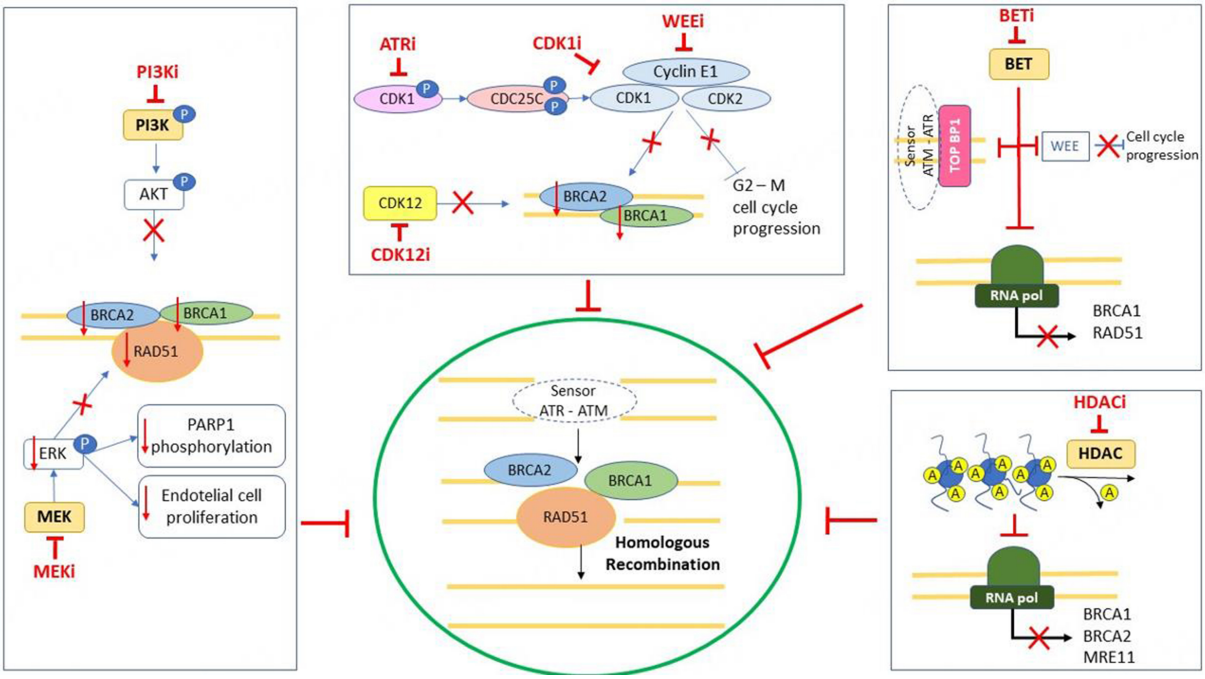

Figure 2. Cellular pathways that can be targeted to overcome PARPi resistance in HRp tumors. ATM: Ataxia-telangiectasia mutated; ATR: ataxia telangiectasia and Rad3-related; CDK1: cyclin-dependent kinase 1; CDK2: cyclin-dependent kinase 2; CDK12: cyclin-dependent kinase 12; ERK: extracellular signal-regulated kinase; HDAC: histone deacetylases; MEK: MAPK/ERK kinase; PARP: poly (ADP-ribose) polymerases inhibitors; PI3K: phosphatidylinositol-3 kinase; RNApol: RNA polymerase; TOPBP1: DNA topoisomerase II binding protein 1.

downregulation seems to be due to ERK-dependent activation of the erythroblast transformation specific (ETS) transcription factor, which inhibits *BRCA1* or *BRCA2* transcription, thereby resulting in HRD and

concomitantly increasing the sensitivity to PARPi[80]. A phase 1b trial investigated the synergy between the PI3Ki alpelisib and olaparib in patients with breast and ovarian cancer. This study included 30 women with EOC (mainly HGSOC) and 93% had platinum resistant/refractory disease. This trial showed an ORR of 33% in *BRCA*wt platinum-resistant patients, while the ORR of olaparib or other PARPi as monotherapy in the same setting ranges from 3% to 10%[89]. An ongoing phase 3 trial is investigating alpelisib and olaparib versus chemotherapy of physician's choice in patients with platinum-resistant *BRCA* wild type EOC (NCT04729387).

MEK inhibition decreased both the MRE11 and *RAD51* foci at DSBs as well as the *BRCA1* nuclear localization, with a consequent accumulation of DNA damage[81]. Moreover, the increased expression of PARP1 and the decreased vascularity increases the hypoxia[81]. It was shown that hypoxia induces transcriptional repression of *BRCA1* expression, with a consequent HRD status sustaining a greater sensitivity to PARPi[90]. Olaparib in combination with selumetinib has been investigated in an early-phase trial in patients with KRAS/NRAS mutant and *BRCA*wt solid tumors, including gynecological cancers. Among 12 evaluable patients, ORR was 17%, and clinical benefit rate (CBR) 33%[91]. Further investigations are needed to further understand the clinical activity of this combination in recurrent EOC.

Cell cycle check-point inhibitors

An effective suppression of HR and a consequent sensitization to PARPi can also be achieved through the inhibition of CDK. Inhibition of CDK12 causes a reduced expression of *BRCA1*, *BRCA2*, and *RAD51*[92]. Inhibition of CDK1 blocks the DNA repair mechanism sustained by the HR pathway and selectively sensitizes cells to PARPi[84]. Xia *et al.* demonstrated a synergistic effect of CDK1 and PARP inhibitors in breast cancer cells proficient for BRCA[93]. Prexasertib is a checkpoint kinase 1 inhibitor (CHK1i) that showed efficacy in a phase 2 trial that enrolled 28 women with *BRCAwt* high-grade EOC. The majority (80%) had platinum-resistant or refractory disease. 8/24 evaluable patients achieved a PR (33%), and the median duration of treatment was 11.4 months[94]. Notably, CCNE1 amplification or copy number gain was detected in half of the patients who achieved a response. Prexasertib also showed early signs of activity when added to olaparib in patients with HGSOC previously treated with a PARPi[95].

PARPi were also evaluated along with WEE1 kinase inhibitors in preclinical models and in clinical trials. The WEE1 kinase prevents entry into mitosis by inhibiting CDK1 and CDK2. WEE1 inhibition causes CDK1 activation, resulting in cell cycle acceleration, early mitotic entry and mitotic catastrophe, particularly when combined with DNA damaging agents[82]. Simultaneous inhibition of PARP and WEE1 is highly toxic, but this can be mitigated by adopting a sequential treatment strategy. Normal cells have low replicative stress and DNA damage, so sequential therapy is effective for tumor cells but less toxic to normal cells[96]. In a recent clinical trial, the efficacy of adavosertib alone or in combination with olaparib was investigated in 80 patients with EOC and PARPi resistance. Patients treated with the combination have a greater CBR (89% *vs.* 63%) compared to WEEi alone, but the ORR was similar between the two arms (29% *vs.* 23%). Among exploratory analyses, *BRCA* mutations appeared to correlate with lower ORR (20% in the adavosertib-alone arm and 19% in the adavosertib/olaparib arm) compared to the *BRCAwt* subgroup (31% in the adavosertib-alone arm and a 39% in the adavosertib-olaparib arm). Data on the benefit according to the HRD status are not yet available. Translational analyses are ongoing to identify potential predictive biomarkers[97].

A synergistic activity among PARPi and ATR inhibitors (ATRi) has been observed in different models of ovarian cancer[98,99]. G2-M checkpoint is lost with ATR inhibition, and as a result, cells with damaged DNA can proceed into the cell cycle, and run into mitotic catastrophe and apoptosis. In addition, PARP inhibition increases dependence on ATR/CHK1 to maintain genome stability[98]. A study in *BRCAwt*,

CCNE1 amplified platinum-resistant ovarian cancer patient-derived xenograft (PDX) models showed that the combination of PARPi and ATRi results in tumor reduction and a significant increase in OS[98]. A phase 2 trial investigated the combination of olaparib and the ATRi ceralasertib to overcome PARPi resistance. In 13 patients with PARPi resistance, an ORR of 46% was observed with a PFS of 7.6 months[99]. Given these preliminary results, future clinical trials are also needed to investigate this combination in HRp EOC as a strategy to overcome intrinsic resistance to PARPi. Moreover, dose optimization trials are required to define the best schedule to overcome the important challenge of the hematological toxicity of the combinations of DDR and cell cycle checkpoint inhibitors.

Histone deacetylases (HDACs) and bromodomain and extraterminal (BET) proteins are involved in gene transcription. BET inhibition downregulates WEE and decreases the expression of TOPBP1, a protein required for the activation of ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). Phosphatidylinositol-3 kinase (PI3K) inhibition decreases *BRCA1* expression, while MEK inhibition decreases RAD51foci. In addition, MEKi increases PARP1 expression and decreases angiogenesis. The inhibition of CDK12 and CDK1 decreases the expression of proteins involved in the homologous recombination. WEE and ATR inhibition results in the loss of the G2-M checkpoint, and cells with damaged DNA advance in mitotic phase. All these mechanisms lead to homologous recombination deficiency.

TARGETING REPLICATION STRESS TO TREAT HRp TUMORS

CCNE and MYC amplified EOC have been identified as possible targets of RS response inhibitors, such as CHK1, ATRi, and WEE1 inhibitors (WEE1i). The inhibition of these checkpoints leads to inappropriate initiation of replication from multiple origins, depletion of replication factors, fork collapse, and cell death[100]. In CCNE1-amplified HGSOC cell lines, the CDK2 inhibitor dinaciclib suppresses cell growth and cell cycle progression, inducing apoptosis with a rise of intracellular radical oxygen species (ROS) levels[101]. The combination of a CDK2i and an AKTi also resulted in tumor regression in CCNE1-amplified cell lines of HGSOC[102]. In MYC overexpressing ovarian cancer xenograft models, the CDK4/6i, palbociclib induced tumor regression when combined with olaparib, showing a potential synergy among these two DDR targeting agents[103].

Among the genes identified as related to a loss of fitness in CCNE amplified cells, there is PKMYT1, which encodes the protein kinase Myt1, involved in the negative regulation of CDK1. RP-6306, a highly selective inhibitor of this target, was tested on several ovarian carcinoma cell lines and showed greater toxicity in CCNE1 amplified preclinical models alone or with gemcitabine[104]. Following these promising preclinical results, RP-6306 enters clinical investigation as a single agent (NCT04855656) and in association with gemcitabine (NCT05147272).

The ATRi berzosertinib has been combined with gemcitabine in a phase II trial in patients with recurrent EOC and a platinum-free interval < 6 months (n = 70). PFS was improved in the experimental arm and the greatest benefit in PFS was obtained in patients with a platinum-free interval ≤ 3 months (PFS 27.7 weeks *vs.* 9 weeks, HR 0.29; 90%CI 0.12 to 0.71)[105]. A preplanned exploratory analysis revealed a better response trend with gemcitabine alone in patients in the Signature 3-negative subgroup (reflective of HRp tumors) compared to patients in the Signature3-positive subgroup (reflective of HRD tumor)[106]. In addition, tumors with high RS achieved a better response to gemcitabine, likely due to the gemcitabine-induced RS through inhibition of DNA repair and suppression of ribonucleotide reductase[106].

Another promising agent in this setting is the WEE1i adavosertib, which has been investigated in different phase 2 trials in platinum-sensitive and resistance recurrent OC[97,107-109]. Interestingly, the preliminary results of a phase 2 study of single-agent adavosertib in CCNE1 overexpressed recurrent platinum-resistant or refractory HGSOC has been presented. The WEE1 inhibitor resulted in a high ORR (53%) and a clinical benefit rate of 61%, with some patients showing sustained responses over time. Translational analyses are ongoing to better define potential predictive biomarkers of WEE1i activity[110].

CONCLUSIONS

Targeting the HRR pathway through PARP inhibition has revolutionized the treatment of EOC. Clinical trials have proved that the HRp subgroup has a modest advantage with PARPi compared to the HRD population. Different genomic assays are now available to assess the presence of HRD. Although in most studies, HRD status detected by these assays has been shown to be able to discriminate the magnitude of PARPi benefit in *BRCA*wt EOC, these assays cannot reliably identify the group of HRp women with EOC that definitively derive no benefit from PARPi. HRD tests and mutational signatures, although promising, do not represent the dynamic changes in the functional status of HRD[111].

Although a direct comparison within a randomized trial is currently lacking, the benefit of PARPi as first-line maintenance is comparable to that observed with bevacizumab in women with HRp EOC. Given that in the HRp population, no benefit was shown in adding olaparib to bevacizumab in first-line settings, bevacizumab could still represent a viable alternative for this subgroup of patients[15]. Pending further data from ongoing clinical trials comparing PARPi *vs.* antiangiogenic treatment in the HRp population, the decision on the best first-line strategy should be based on the toxicity profile of the two classes of drugs and patients' comorbidities.

Different studies are ongoing to explore strategies to induce HRD and increase the sensitivity to PARPi, even in HRp tumors. Recently, new insights into the biology of HRp and platinum-resistant tumors are sustaining the development of new promising agents targeting the RS[100]. These new agents, alone or in combination, have shown preliminary signs of activity, but their use is challenged by the safety profile and the need to define optimal doses and schedules to maximize the clinical activity while minimizing the occurrence and severity of the adverse events.

DECLARATIONS

Authors' contributions

Contributed to the conception and design of the manuscript: Zielli T, Colombo I
Wrote the first draft of the manuscript: Zielli T, Colombo I
Contributed to literature analysis: Zielli T, Labidi-Galy I, Del Grande M, Sessa C, Colombo I
All authors contributed to the manuscript revision, read and approved the submitted version.

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Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Primary and acquired resistance to first-line therapy for clear cell renal cell carcinoma

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Abstract

The introduction of first-line combinations had improved the outcomes for metastatic renal cell carcinoma (mRCC) compared to sunitinib. However, some patients either have inherent resistance or develop resistance as a result of the treatment. Depending on the kind of therapy employed, many factors underlie resistance to systemic therapy. Angiogenesis and the tumor immune microenvironment (TIME), nevertheless, are inextricably linked. Although angiogenesis and the manipulation of the tumor microenvironment are linked to hypoxia, which emerges as a hallmark of renal cell carcinoma (RCC) pathogenesis, it is only one of the potential elements involved in the distinctive intra- and inter-tumor heterogeneity of RCC that is still dynamic. We may be able to more correctly predict therapy response and comprehend the mechanisms underlying primary or acquired resistance by integrating tumor genetic and immunological markers. In order to provide tools for patient selection and to generate hypotheses for the development of new strategies to overcome resistance, we reviewed the most recent research on the mechanisms of primary and acquired resistance to immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs) that target the vascular endothelial growth factor receptor (VEGFR).We can choose patients' treatments and cancer preventive strategies using an evolutionary approach thanks to the few evolutionary trajectories that characterize ccRCC.

Keywords: Renal cell carcinoma, resistance, tumor microenvironment, checkpoint inhibitors, target therapy

INTRODUCTION

The recent approval of the new first-line combinations, which include immune checkpoint inhibitors (ICIs) both plus VEGFR-TKIs or the anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) monoclonal antibody (mAb), ipilimumab, has revolutionized the treatment of metastatic renal cell carcinoma, reporting improved outcomes in pivotal studies[1-7]. Despite the excellent response rates, some patients are either innately resistant to therapy or eventually develop later resistance to it. Hence, a better understanding of the mechanisms underlying VEGFR-TKI and/or ICI resistance will be helpful in selecting patients who might not respond to this kind of approach and developing strategies to overcome resistance.

Today, the only validated risk assessment tool is risk stratification according to the International mRCC Database Consortium (IMDC) score, which is based upon six clinical and laboratory features[8,9]. However, this approach lacks the ability to recognize genetic and intrinsic factors that potentially direct response to immunotherapy and has only been thoroughly validated for patients treated with single agent VEGFR-targeted therapies.

Here, we reviewed the most recent research on the factors that contribute to both primary and acquired resistance to VEGF-TKI and ICI with the aim of supplying tools for patient selection and generating hypotheses in an effort to decrease the proportion of patients who do not respond or to postpone the emergence of resistance.

MOLECULAR SUBSETS IN METASTATIC RENAL CELL CARCINOMA

Clear cell renal cell carcinoma (ccRCC) is a highly inflamed and immune-infiltrated tumor type with high expression of immune checkpoints, such as PD-L1 and CTLA-4. However, ccRCC has the peculiarity of having a high degree of infiltration by exhausted CD8+ tumor-infiltrating lymphocytes (TILs), immunosuppressive cells such as M2-like tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), which characterized a tumor microenvironment with immunosuppressive properties[10-12].

Immune cells (IC) are components of tumor immune microenvironment (TIME) and play an important role in modulating immune response to tumor cells[13]. These cells could have been implicated both in immune tumor suppression and tumor escape.

More than just identifying individual cells, the microenvironment's composition could provide insight into the mechanisms causing immune escape, and choosing more effective targets could lead to better results. Using ccRCC samples, Chevrier *et al.* examined the TIME's composition and discovered a particular exhausted CD8+/PD-1+ T cell phenotype that was defined by the co-expression of inhibitory receptors and might be the cause of immune suppression. Additionally, they discovered CD38 to be a marker of exhaustion in the CD8+/PD-1+ T cell phenotype, and these cells were closely associated with the presence of regulatory CD4+ T cells and of a cluster of macrophages with the highest expression of CD38 and immune suppressive activity.

Rather than focusing on each cell individually, TIME composition and the number of immune cells may be able to predict outcomes more accurately[14].

Additionally, the TIME could be altered by the use of VEGFR-TKIs and ICIs[15-21], and the TIME may also be impacted by genetic changes in ccRCC, such as von Hippel-Lindau (VHL) and PBRM1mutations. As a result, different genomic signatures may confer a different response to a specific treatment. Therefore, a

deeper comprehension of the molecular traits that uniquely distinguish ccRCC is required to enhance patient selection, risk stratification, and resistance mechanism definition.

Three major gene expression signatures have been identified using data from the first-line pivotal trial in mRCC (IMmotion 150, JAVELIN RENAL 101, Checkmate 214): Angiogenesis, T-effector (Teff)/IFN-γ response, and myeloid inflammatory gene expression signatures[22-24].

Gene expression patterns that had been previously described in relation to their corresponding biology were used for the definition of gene signatures. Angio: VEGFA, KDR, ESM1, PECAM1, ANGPTL4, and CD34; Teff: CD8A, EOMES, PRF1, IFNG, and CD274; myeloid inflammation: IL-6, CXCL1, CXCL2, CXCL3, CXCL8, and PTGS2[25-27].

Angio gene profile identified a group of patients who would respond to a VEGFR-TKI alone, resulting in superior outcomes with sunitinib in each study. Given that a high T effector gene signature may indicate an improved response to a VEGFR-TKI and ICI combination therapy, the presence of a myeloid signature in the T effector high group identified tumors that are resistant to immunotherapy when used alone, as demonstrated by the worse outcomes for patients treated with atezolizumab alone in the IMmotion 150 trial. Furthermore, a myeloid infiltrate, which is indicative of innate resistance to immunotherapy alone, could be overcome by the addition of a therapy targeting angiogenesis. Gene signatures based on a single class of genes may be effective tools that support patient selection. However, when using two immune checkpoint inhibitors, a combination of gene signatures including both innate and adaptative immune response components may be more suggestive of how TIME and therapies interact and may be more predictive of results. The "Renal 101 Immune signature", a 26-gene subset of the gene expression signature (GES) that included regulators of innate and adaptive immunological responses (T cell and NK cell), cell trafficking, and inflammation, identified in JAVELIN RENAL 101, is an even more comprehensive molecular predictive tool, underlying the significance of CD8+ T cells in inducing immune response[23].

Table 1 schematically summarizes the correlation of GES (Angio, Teff, Myeloid) with positive outcomes in IMmotion 150, Javelin RENAL 101, and Checkmate 214.

MOLECULAR SUBSETS IN RCC AS BIOMARKER STRATEGIES FOR PERSONALIZED TREATMENT

In 2020, McDermott *et al.* completed a significant research effort to evaluate the outcomes of patients who received a combination of checkpoint inhibitors, applying the previously developed IMmotion 150 signatures to the IMmotion 151 trial[22]. In order to develop a new molecular categorization of RCC, they performed an integrative multi-omics analysis of 823 RCC tumors[28,29].

Non-negative matrix factorization (NMF) was used to identify seven distinct molecular clusters, and the distribution of these clusters across IMDC risk groups was assessed. They examined the somatic alterations within each cluster and investigated the clinical outcomes of patients who received atezolizumab in combination with bevacizumab, sunitinib, and atezolizumab across clusters.

Table 2 synthetizes cluster characteristics, gene profile expression, and their correlation with outcomes.

Angiogenic clusters (1 and 2) were enriched in the favorable risk group (evaluated both according to MSKCC and IMDC risk categories) and showed better progressive-free survival (PFS). However, angiogenic

Table 1. Correlation between GES with favorable outcomes

	IMmotion 150[22]			Javelin renal 101[23]		Checkmate 214[24]	
	Atezo + Beva	Atezo	Sun	Ave + Axi	Sun	Nivo + Ipi	Sun
$Angio^{high}$			√		√		√
$Angio^{low}$	√						
$Teff^{high}$	√			√			
$Teff^{low}$			√				
$Myeloid^{low}$	√	√					
$Myeloid^{high}$			√				
$Teff^{high}$ $Myeloid^{high}$	√						
$Teff^{high}$ $Myeoid^{low}$		√				√	
26-gene immune signature				√			
26-gene immune signature					√		

Angio: Angiogenic; Atezo: atezolizumab; Ave: avelumab; Axi: axitinib; Beva: bevacizumab; GES: gene expression signature; Ipi: ipilimumab; Nivo: nivolumab; Sun: sunitinib; Teff: T-effector.

signature did not differentiate outcomes between arms. No correlation was also seen for patients belonging to cluster 3 (complement/-oxidation).

Otherwise, the poor-risk group was more likely to have proliferative clusters (4-6), with stromal/proliferative clusters demonstrating the shorter PFS irrespective of treatment arm. Teff/proliferative and proliferative cluster results, as well as the snoRNAs cluster, experienced better outcomes with the atezolizumab plus bevacizumab combination.

Among somatic alterations, PBRM1 mutations gave superior results, irrespective of the treatment arm. But in PBRM1 mutant patients, Atezolizumab + Bevacizumab showed improved PFS and ORR than sunitinib. Conversely, CDKN2A/B alterations identified patients with worse prognoses. However, CDKN2A/A-alterated tumors had better PFS and ORR in the atezolizumab + bevacizumab arm compared with the sunitinib arm.

Better outcomes with the combination were also seen for tumors harboring loss-of-function mutations of ARID1A and/or KMT2C[28].

In conclusion, these molecular subsets constitute a novel method of reproducible response prediction that may be useful in patient selection. As shown for clusters 1 and 2, the angiogenesis pathway confers a biological behavior that is comparable for tumors treated with a VEGFR-TKI alone or in combination. A proliferative pattern, on the other hand, suggests a lack of response to a therapy that targets angiogenesis alone and a potentially better response to a combination therapy that includes ICIs. This classification does not include the combination of dual checkpoint inhibitors, which would restrict its reproducibility. However, we are aware that nivolumab + ipilimumab has shown superior outcomes in the category of intermediate-poor risk.

The tumors from patients with favorable risk in this study exhibited a higher expression of the VEGF pathway-associated angiogenesis signature, which provides a potential explanation for why the dual combination failed to improve outcomes in the favorable risk subgroup.

Table 2. Molecular clusters by NMF, gene expression profiles, DNA alterations, and correlation with outcomes

	Cluster 1		Cluster 2		Cluster 3		Cluster 4		Cluster 5		Cluster 6		Cluster 7	
Name	Angiogenic/ Stromal		Angiogenic		Complement/ -Oxidation		Teff/ Proliferative		Proliferative		Stromal/ Proliferative		snoRNA	
Transcriptional Pathways	Angiogenesis Stroma		Angiogenesis Catabolic metabolism (FAO)		Complement cascade -oxidation		Cell-cycle Teff Anabolic metabolism (FAS)		Cell-cycle Anabolic metabolism (FAS) Myeloid inflammation		Cell-cycle Stroma		snoRNAs	
Gene expression module	TGF, WNT, Hedgehog, NOTCH		TGF, WNT, Hedgehog, NOTCH		Complement cascade		Cell-cycle		Cell-cycle		Cell-cycle		C/D box snoRNAs (SNORDs)	
	Stroma-genes: Fibroblast-derived genes *FAP, FN1, PSTN, MMP2*		Catabolic mb: *FAO/AMPK* genes		Cytochrome P450 family		Anabolic mb FAS Pentose phosphate		Anabolic mb		Anabolic mb Stroma-genes			
			Moderate expression: Teff genes		*Moderate expression: Cell-cycle genes*		Teff JAK/ STAT IFN-α and -γ		Myeloid inflammation genes		EMT transcriptional Myeloid inflammation genes			
DNA alterations	PBRM1 VHL KDM5C PTEN		VHL PBRM1 KDM5C		VHL PBRM1 KDM5C PTEN BAP1		VHL CDKN2A/B BAP1		CDKN2A/B TP53 TFE fusions (mTORC1 pathway)		VHL CDKN2A/B TP53		VHL SETD2 PTEN	
PFS Months HR (95%CI)	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun
	15.3	13.9	13.8	14.2	8.1	7.1	10.9	6.1	8.3	4.3	6.8	5.2	NR	7.4
	HR 1.11 (0.65-1.88) *P* = 0.708		HR 1.16 (0.82-1.63) *P* = 0.397		HR 0.92 (0.63-1.34) *P* = 0.666		HR 0.52 (0.33-0.82) *P* = 0.005		HR 0.47 (0.27-0.82) *P* = 0.007		HR 0.81 (0.52-1.25) *P* = 0.331		HR 0.10 (0.01-0.77) *P* = 0.028	
OS Months HR (95%CI)	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun	Ate/bev	Sun
	NR	48.2	46.2	NR	35	36.6	38.7	23.3	21.7	15.5	15.9	12.7	NR	NR
	HR 0.94 (0.52-1.72)		HR 1.32 (0.91-1.91)		HR 0.99 (0.64-1.54)		HR 0.66 (0.41-1.06)		HR 0.66 (0.39-1.12)		HR 0.90 (0.57-1.40)		HR NC	

AMPK: Activate protein kinase; Ate: atezolizumab; Bev: bevacizumab; FAO: fatty acid oxidation; FAS: fatty acid synthesis; Mb: metabolism; Sun: sunitinib; TGF: tumor growth factor beta.

MECHANISMS OF PRIMARY RESISTANCE TO TYROSINE KINASE INHIBITOR IN RENAL CELL CARCINOMA

Primary resistance is characterized as a lack of response to therapy, which may be caused by the absence of a particular target's expression or by the existence of inherently resistant clones that do not respond to target therapy. Furthermore, primary resistance may be influenced by spatial and temporal heterogeneity[30,31].

Primary resistance to VEGF-TKIs

Hypoxia and von hippel lindau pathway

The tumor suppressor protein von Hippel Lindau (VHL) is frequently mutated in hereditary RCC. VHL is a target of hypoxia-inducible factors (HIFs), dimeric proteins composed of O2-sensitive subunits (HIF-1, -2 or 3) and a subunit (HIF-2). In the presence of oxygen, these factors facilitated its degradation. VHL inactivation creates a pseudo-hypoxic state and HIF dimers can bind to hypoxia response elements (HREs) to induce angiogenesis and cancer cell proliferation. VHL disease is characterized by a decreased expression of HIF-1 and an increased expression of HIF-2, the latter connected with c-Myc activity[32].

Gordan *et al.* analyzed 160 tumor samples and found that VHL-deficient ccRCCs can be distinguished based on HIF- expression. Three different subgroups have been defined: (1) Wild-type VHL tumors with no HIF- expression; (2) VHL deficient tumors with HIF-1 and-2 expression; and (3) VHL deficient tumors with only HIF-2. The third subgroup (HIF-2 expression) displayed enhanced c-Myc activity and higher rates of proliferation. The authors also demonstrated an interplay between HIF-2, c-Myc and genome instability. Indeed, the VHL-deficient subgroup expressing HIF-2 was even characterized by an upregulation of the homologous recombination (HR) effectors BRCA1 and BARD1, and consequentially, HIF-2 tumors and no HIF-1 ones had a greater ability to repair DNA damage accumulation induced by replication stress[32,33].

Given that VHL-deficient tumors with only HIF-2 expression experienced primary resistance to angiogenesis inhibition, thus HIF-2a alone may identify a subset of RCCs in which targeted therapies lack efficacy.

Membrane transporters and lysosomal sequestration

The uptake and efflux of several TKIs (e.g., sunitinib, cabozantinib, pazopanib) could be mediated by multidrug resistance (MDR)-related solute carrier (SLC) and ATB-binding cassette (ABC) transporters, respectively[34,35]. A number of variables, including pH, drug concentration, and affinity, can affect the interaction between TKIs and transporters. As a result of insufficient intracellular drug concentration, these transporters can be responsible for intrinsic resistance.

The most studied ABC transporters implicated in MDR include P-glycoprotein (Pgp, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2). Due to a substrate-like characteristic, TKIs can be pumped outside the cells with an efflux mechanism at lower concentrations. However, at higher concentrations, TKIs can act as inhibitors.

TKIs usually inhibit ABC transporters without altering their expression or localization. As an example, cabozantinib competitively interacts with the drug-substrate binding site to decrease the ATPase activity of the ABCG2 transporter[36]. Pazopanib has both substrate-like and inhibitory effects. In the canine kidney cell line MDCKII, it was reported as both an ABCB1 and ABCG2 substrate and as an inhibitor of ABCB1 and its efflux characteristics[37-39].

Lysosomal sequestration is another mechanism of resistance based on drug physicochemical properties. This phenomenon is related to ABC transporters since these pumps are present on the membranes of intracellular compartments and regulate the drug's influx into lysosomes[37,40,41]. Lysosomal sequestration has been shown to impact the effectiveness of the drugs sunitinib and pazopanib[42].

Lysosomal intake-induced resistance can be reversed. Indeed, after removing sunitinib from tumor cell culture, cell lysosomal capacity was restored, regaining drug sensitivity. This can give an explanation of the recovered sensibility to sunitinib experienced by patients after treatment interruption and subsequent rechallenge[37,42,43].

SECONDARY AND ACQUIRED RESISTANCE TO VEGFR-TKIs

Bergers and Hanahan categorize resistance to VEGFR-TKI as intrinsic or primary and adaptative or evasive (secondary)[44]. Sometimes, it was impossible to clearly and immediately shift the biological basis of these two types of resistance. The majority of the time, primary resistance could be explained by the abundance of angiogenic receptors and downstream pathways. However, the hypoxic state induced by VEGFR-TKI therapy could be responsible for an "angiogenic switch" towards a different molecular pathway, driving a different pattern of response [Figure 1].

Acquired resistance develops throughout treatment, usually following an initial response to therapy, and could be induced by the pressure of a specific therapy. The selection of particular clones that are resistant to therapy results in the progression of cancer. Additionally, cancers might develop resistance to a particular treatment through other pathways that are not affected by targeted therapy.

Tumor plasticity

A loss of cell polarity and contact promotes epithelial-mesenchymal transition (EMT). E-cadherin and other epithelial cell markers are downregulated during this transition, while mesenchymal markers including N-cadherin, vimentin, fibronectin, different matrix metalloproteases (MMPs), and 1 and 3 integrins are upregulated[45,46]. It has been shown that HIF-1 activation caused by hypoxia can induce EMT, a process that is associated with drug resistance[47-51]. EMT-associated transcription factors (EMT-TFs), such as TWIST1, ZEB1, or SNAI1, can be stimulated to express themselves directly or indirectly by the stabilization of HIFs under hypoxia. TGF/TGFR, NF-B, and NOTCH signaling are the three regulatory mechanisms most extensively studied for their potential role in triggering EMT[52-55].

Insulin-growth factor (IGF) signaling, which interacts with the NOTCH and Wnt/-catenin pathways, has been shown to be a modulator of EMT[56].

Sharma *et al.* provided evidence that sunitinib-treated RCC tumors underwent a mesenchymal transformation as seen by higher expression of N cadherin and decreased expression of E cadherin, which were both connected with an elevation of TGF and IGF1R. Patients with mRCC who expressed IGF1R and TGF and subsequently had EMT had worse outcomes, as shown by data from the Cancer Genome Atlas (TCGA) database[57].

Hwang *et al.* analyzed gene expression profiles and copy number variations of 10 metastatic ccRCC tumor samples before treatment and immediately after disease progression to a TKI. Microarray analysis of pre- and post-treatment ccRCC tumors demonstrated an increased expression of EMT-related genes including CD44, SNAI2, TWIST, and CLDN1 in TKI-resistant cells, acquiring migration and invasion capacity. In this study, the authors demonstrated that CD44 depletion significantly decreased cell invasiveness.

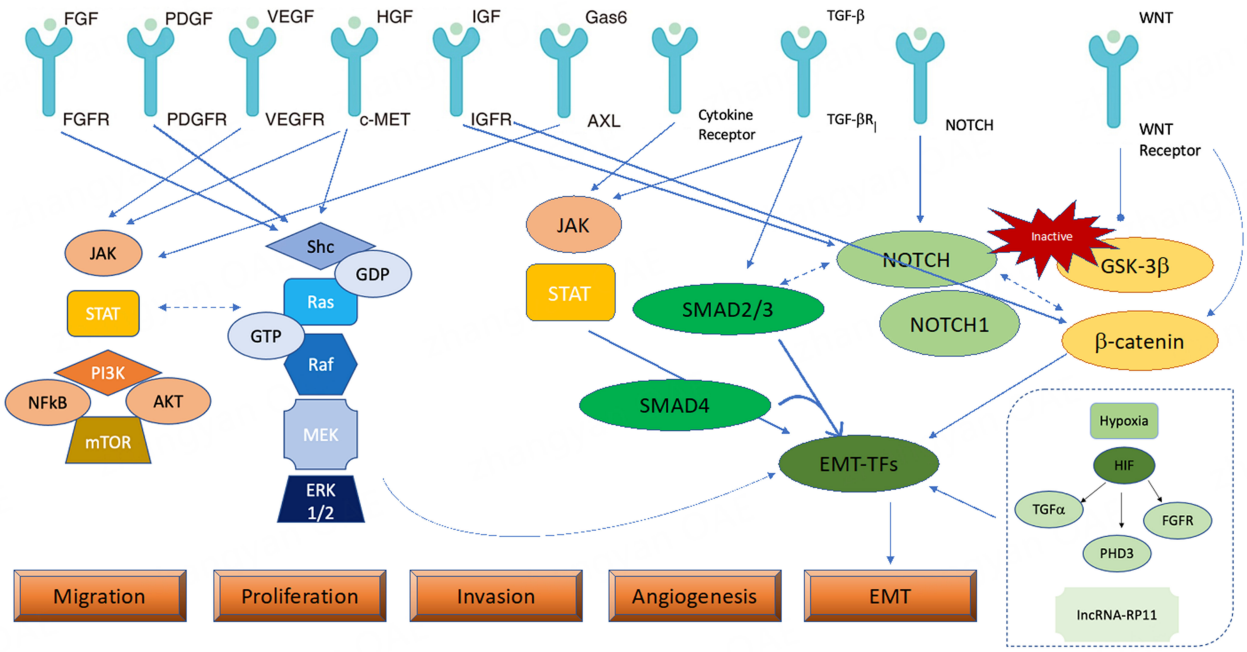

Figure 1. Cross-talking between TKI receptors, hypoxia and growth factor receptors and relationship with tumor growth and resistance to TKIs. Several trans-membrane TKI receptors interact with each other and mediate the activation of shared pathways, most implicated in tumor growth and angiogenesis. TGFβ/Smad pathway and the interaction of some cytokines, such as IL-6 with their receptors, together with the Notch signaling pathway and wnt/β-catenin pathway induce EMT transcription factors and EMT as a mechanism of resistance to the inhibition of angiogenesis. Finally, hypoxia promotes both angiogenesis and EMT. AKT: Protein kinase B; AXL: AXL Receptor; EMT: epithelial mesenchymal transition. EMT-TFs: epithelial mesenchymal transition transcription factors; Erk1/2: elk-related tyrosine kinase; FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; Gas6: growth arrest specific protein-6; GSK-3: glycogen synthase kinase 3 beta; HGF: hepatocyte growth factor; HIF: hypoxia-inducible factor; IGF: insulin growth factor; TGF-: tumor growth factor; IGFR: insulin growth factor receptor; JAK: janus kinase; Mek1/2: MAP kinase-ERK kinase; MET: hepatocyte growth factor receptor; mTOR: mammalian target of rapamycin; NFκB: nuclear factor kappa B; PDGFR: platelet derived growth factor receptor; PDGF-β: platelet derived growth factor-β; PI3K: phosphatidyl inositol 3-kinase; Raf: RAF proto-oncogene serine/threonine-protein kinase; Ras: rat sarcoma protein; STAT3: signal transducer and activator of transcription 3; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; VHL: von hippel-lindau protein.

Claudin-1 is a component of tight junctions, and it has been suggested that it could be implicated in EMT induction. In this study, claudin-1 expression seems to be a mediator of TKI resistance in both clinical and experimental models. Furthermore, compared to pre-treatment ccRCC, TKI-resistant tumors had an increased proportion of sarcomatoid features[58].

It may be crucial to understand the various pathways that underlie EMT and the indicators of this process in order to design strategies that combine therapies addressing both hypoxia and EMT.

Non-angiogenic pathway and by-pass pathways

Vascular co-option is a way to use pre-existing vessels. Using 164 lung metastasis specimens, Bridgeman *et al.* identified four different histopathological growth patterns (HGPs), each with a different vascularization (alveolar, interstitial, perivascular cuffing, and pushing). The tumors vascularize through angiogenesis exclusively in the pushing HGP pattern; a co-option vascular mechanism was employed in the other patterns. The authors showed that vascular co-option might act as a mediator of sunitinib resistance. In mice models, sunitinib induced a switch from the most frequent angiogenic pushing HGP to an alveolar/interstitial HGP that vascularizes through vessel co-option, thus inducing resistance[59].

Furthermore, cancer resistance can arise when tumor cells employ other signaling pathways that are unaffected by VEGF/VEGFR suppression.

Fibroblast growth factor/fibroblast growth factor receptor pathway

Fibroblast growth factors (FGFs) are proteins that are involved in proliferation, differentiation, migration, and apoptosis of tumor cells[60].

FGF2, also known as basic FGF, has been recognized as a potential mediator of TKI resistance. Cell cultures were treated with VEGF and 100 nM sunitinib by Welti *et al.* Endothelial cell proliferation was restored by FGF1 and FGF2 to levels that were comparable to (FGF1) or greater than (FGF2) those seen in the absence of sunitinib[61]. Indeed, FGF2 induces angiogenesis through the activation of signaling pathways such as Ras-Raf-MEK-ERK 1/2 and PLC-PKC, bypassing the VEGF/VEGFR signal. It has been reported that FGF2 upregulates the expression of both fibroblast growth factor receptor (FGFR) and VEGFR in endothelial cells and systemic administration of VEGFR-2 antagonists inhibits both VEGF and FGF2-induced angiogenesis in vitro and in vivo[62-64]. In contrast to what was expected by previous studies, Welti *et al.* showed that while sunitinib inhibits VEGFR2-mediated activation of ERK 1/2 and PLC, it is not able to prevent the FGF2-mediated activation of these pathways, raising the possibility that cancer cells may use this way to bypass VEGF-mediated angiogenesis inhibition[61].

MET/HGF signaling

When the hepatocyte growth factor/scatter factor (HGF/SF) binds to its receptor tyrosine kinase MET, the activation of the RAS-MAPK and PI3K-AKT pathways results in the development and angiogenesis of endothelial cells. HGF/SF-MET interaction is a potent regulator of the angiogenic switch. Common signaling intermediaries such as ERK-MAPK, protein kinase B (AKT), and focal adhesion kinase (FAK) are activated by both MET and VEGFR[65-68].

A remarkable new finding is that the MET/HGF pathway may be activated by hypoxia caused by blocking angiogenesis with VEGFR-TKIs in a HIF-mediated manner, hence increasing the MET-dependent spread of cancer cells[69-72].

Additionally, it is hypothesized a relationship between the MET axis and the immune system. Several immune cells, including mast cells, neutrophils, and dendritic cells (DCs), may have increased MET expression. MET/HGF-SF signaling could impact the ability of T cells to respond competently to cancer cells, by reducing the DCs' capacity to present antigens and recruiting immunosuppressive cells. Therefore, MET inhibition may be a way to restore neutrophils and DCs' capacity[73,74].

Cabozantinib is an oral multiple tyrosine kinase receptor inhibitor: VEGFR2, c-MET, and RET. Inhibition of VEGFR and c-MET decreases resistance to VEGFR inhibitors via the c-MET axis. However, resistance to MET inhibition can occur. Huang *et al.* reported some of the most common mechanisms inducing acquired resistance to HGF/MET-target therapy: hypoxia-induced MET phosphorylation reduction, with no effect on downstream signaling pathway, mutations in the MET kinase domain, bypass signaling, copy number changes and constitutive activation of AKT and ERK-MAPK pathway[73,75-77].

GAS6/Axl signaling

AXL is a receptor tyrosine kinase (RTK) that is a member of the TAM RTK. AXL signaling is implicated in tumor growth, EMT, angiogenesis, metastasis spread, and the development of resistance to targeted therapy. The AXL-ligand Gas6 is a vitamin K-dependent protein and the GAS6/AXL signaling can be constitutively

activated in ccRCC cells[78-82].

Gustafsson *et al.* found that sunitinib enhanced Gas6-induced AXL phosphorylation in ccRCC, and consequentially activated the AKT pathway. Indeed, in the absence of sunitinib, the activation of the main MAPK pathways (ERK1-2, P38MAPK, and SAPK-JNK) by Gas6 alone was insufficient to activate AXL. Furthermore, it was shown that Gas6 activated the EGFR pathway when sunitinib was present. This pathway, along with AXL, is thought to be implicated in cancer's resistance mechanism[83].

Cytokines

Treatment with sunitinib has been shown to increase the expression of IL-6 and IL-8. These cytokines have been linked to TKI resistance, suggesting that they could play an important role in inducing angiogenesis in a HIF-independent way. IL-6 activates the AKT/mTOR and transcription factor STAT3 cascade, resulting in increased expression of VEGFA and VEGFR2. In endothelial cells, IL-8 promotes the accumulation of VEGFA mRNA in normoxic conditions as well as the endothelial cells are exposed to hypoxia. Even when HIF-1 is blocked, CXCL8/IL-8 can still induce VEGFA promoter-driven transcription[84,85].

Pilskog *et al.* evaluated the expression of IL6R in RCC tumor cells and discovered that the expression of IL6Ra may predict responsiveness to TKI treatment. In fact, a significant correlation between IL6R expression and the objective response rate was identified but not with PFS or OS, indicating that IL6R expression may have predictive significance. IL-6 ligand expression may also play a prognostic role, as demonstrated by the association between its lack of expression or low expression with PFS[86,87].

Huang *et al.* developed sunitinib-resistant xenograft models and discovered that sunitinib-treated ccRCC cell lines developed resistance and displayed an elevated IL-8 expression. They observed that only when sunitinib treatment was sustained over a longer period did the reduction of IL-8 function decrease tumor growth. Only after the emergence of resistance to tyrosine kinase inhibition could IL-8 function inhibition affect tumor growth[88,89].

Tumor microenvironment and immune cells as mediators of TKI-resistance

There is growing interest in the connection between TIME, immune cells, and angiogenesis, and there is evidence that the tumor microenvironment has a direct role in the emergence of resistance to targeted therapies. The recently approved combination of ICIs and VEGFR-TKIs is also supported by this association. Therefore, it is critical to understand how TIME affects the mechanisms of resistance to target therapy in order to better understand resistance to combination therapy [Figure 2].

MDSCs

MDSCs are the major component of TIME. Growing evidence indicates that tumors release pro-angiogenic signals that recruit MDSCs, which may act as mediators in the development of resistance to TKIs[91-94].

Ko *et al.* had demonstrated that sunitinib therapy significantly reduced MDSC accumulation in tumor-bearing mice models, leading to improved peripheral T-cell function. It appears that even when sunitinib diminishes peripheral MDSC accumulation, intra-tumoral MDSCs can be much less impacted. Intra-tumoral MDSCs from sunitinib-treated mice retained T-cell suppressive capacities comparable to those from untreated mice. The authors quantified MDSC subsets in tumor specimens of untreated and sunitinib-treated RCC. In contrast to the substantial reduction in peripheral blood MDSCs seen in RCC patients treated with sunitinib, the proportion of MDSCs in tumor samples nevertheless remained greater and these cells maintained their suppressive capabilities, as assessed by IFN production.

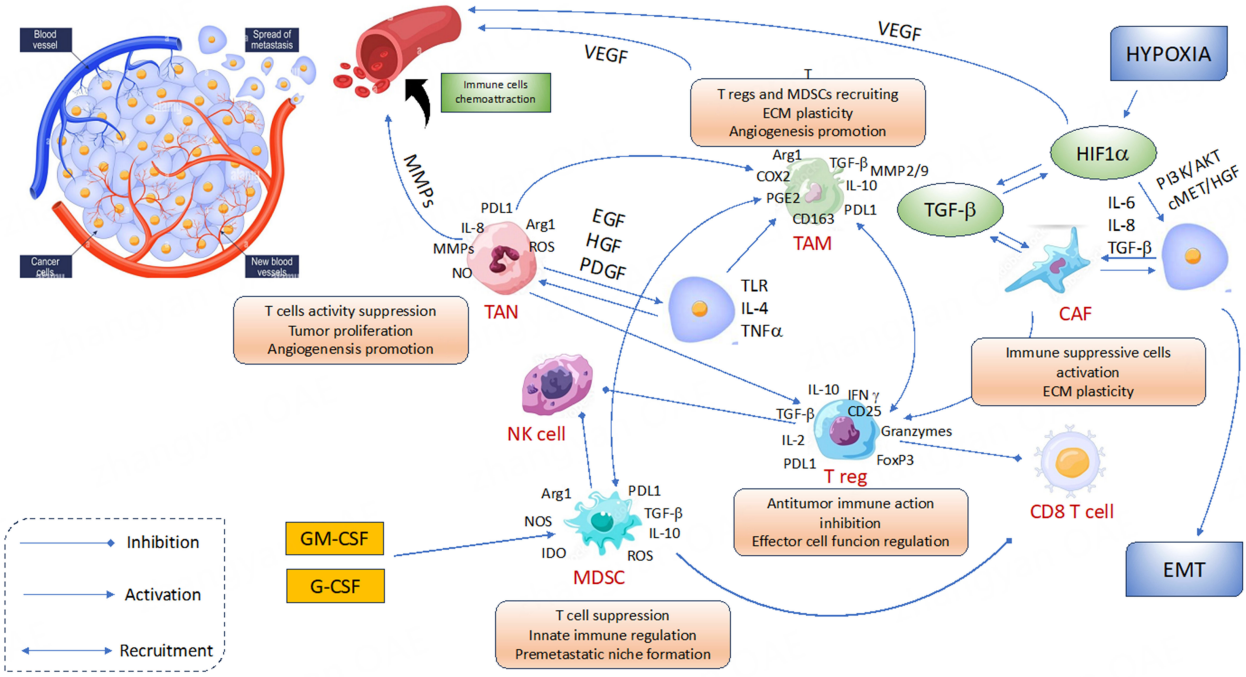

Figure 2. Interaction between angiogenesis and tumor microenvironment. Immune cells are recruited by chemokines and angiogenic factors and the tumor infiltration by immune cells is, in turn, implicated in promoting angiogenesis. The balance between immune response and immunosuppression is crucial to induce tumor killing from one side or tumor escape to the other. CAF: Cancer-associated fibroblasts; EGF: epidermal growth factor; EMT: epithelial-mesenchymal transition; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; HGF: hepatocyte growth factor; HIF-1α: hypoxia-inducible factor 1α; IFNγ: interferon-γ; IL-2/4/6/10/8: interleukin; MDSC: myeloid-derived suppressor cell; MMP: matrix metalloproteinases; NK: natural killer; PDGF: platelet-derived growth factor; PDL1: programmed death-ligand 1; TAM: tumor-associated macrophages; TAN: tumor-associated neutrophils; TGF-β: tumor growth factor beta; TLR: toll-like receptor; TNFα: tumor necrosis factor α; Treg: regulatory T cells; VEGF: vascular endothelial growth factor[90].

Additionally, sunitinib did not significantly affect the amount of GM-CSF produced by RCC tumors in vitro. Due to the stimulation of the STAT5 pathway, the pro-proliferative cytokine GM-CSF confers peripheral MDSCs sunitinib resistance. Sunitinib decreased pSTAT3 in the absence of GM-CSF, which made MDSCs more susceptible to the drug. In contrast, STAT5-mediated pathways are prominent in the presence of GM-CSF, resulting in a phenotype of MDSCs that is resistant to sunitinib[95].

These findings have been confirmed by Finke *et al.*, who examined MSDCs both in peripheral blood and in tumors of RCC patients. They discovered that the sunitinib-induced maximum drop in MDSC numbers occurred after the second cycle and continued until later than the fourth cycle, although there is some recovery in MDSC levels at this point. In this study, the impact of pro-angiogenic factors on sunitinib resistance was also evaluated. Pro-angiogenic variables' effects on sunitinib resistance were also assessed in this study. The analysis of tumor tissue lysates from patients who had neoadjuvant therapy revealed that there was an increase in the expression of pro-angiogenic proteins [Matrix metallopeptidase (MMP9), MMP8, and IL-8] in tumors with a greater level of MDSCs. Additionally, sunitinib causes an increase in plasma IL-8 levels, and larger levels are associated with a worse PFS. The presence of MDSCs in tumors might promote the production of IL-8, which could activate alternative pro-angiogenic pathways (MMP9/MMP8/IL8) to prevent cell death[96].

C Marcela Diaz-Montero *et al.* used a patient-derived xenograft (PDX) model of RCC and performed a microarray analysis of sunitinib-responsive and -resistant tumors. Resistance to sunitinib was associated

with the upregulation of genes involved in cell movement and immune cell trafficking in both human and murine expression analyses. Furthermore, tumors resistant to sunitinib had higher levels of G-CSF and, consequently, higher levels of G-MDSCs. G-MDSCs are MDSCs that resemble granulocytes both phenotypically and functionally. G-MDSCs have the ability to inhibit the immune response, and they can additionally promote angiogenesis and the spread of tumors[97].

Cancer-associated fibroblasts

Tumor-growth factor (TGF) and platelet-derived growth factor (PDGF) induce the transformation of tumor fibroblasts (FHNR) in cancer-associated fibroblasts (CAFs), which play a key role in tumor progression, producing pro-tumoral cytokines (e.g., IL-6, IL-8, TNF, IL-10). Fibroblast-associated protein (FAP) is a marker of CAFs. Ambrosetti *et al.* demonstrated that high levels of FAP mRNA were correlated with shorter PFS and OS in metastatic ccRCC (PFS, $P = 0.054$ and OS, $P = 0.022$). Sunitinib stimulated FHNR to differentiate towards CAFs. Mice with induced mRCC were treated with sunitinib or with placebo. Compared to the placebo group, the sunitinib-treated mice's tumors had higher FAP mRNA levels ($P = 0.049$). Sunitinib primary resistance has also been linked to CAFs, which create a barrier to the drug's ability to reach tumor cells. Finally, CAFs appear to be a mediator of tumor cell EMT[98].

TAMs

Some of the most numerous immune cells identified within tumors are TAMs. Two subsets of TAMs are typically recognized: M1 and M2. M1-like TAMs have a pro-inflammatory phenotype and inhibit tumor growth, whereas M2-like TAMs have tumor-promoting capabilities involving immuno-suppression, angiogenesis, and neovascularization, as well as stromal activation and remodeling[99]. The functional phenotype M2-like is induced by hypoxia of the TIME, resulting in tumor escape[100].

In the genomic and transcriptomic analysis of ccRCC patients treated with TKIs in the COMPARZ phase III trial, Hakimi *et al.* observed significantly worse OS (HR 1.54; 95%CI: 1.17-2.03; $P = 1.98$) among subjects with high macrophage infiltration and higher macrophage infiltration (Kruskal-Wallis test, $P = 0.02$) in patients who experienced progressive disease. Furthermore, they found that a high M2-macrophage infiltration ($M2^{high}$) was associated with poor OS (HR 1.38; 95%CI: 1.06-1.81; $P = 0.019$) and PFS (HR 1.40; 95%CI 1.09-1.78; $P = 7.90$) compared to the $M2^{low}$ group. Depending on the TKI used, macrophage infiltration and its impact on the outcomes differed. TAMs infiltration was a prognostic factor in patients receiving pazopanib but not sunitinib. This supports the notion that sunitinib primarily affects MDSCs[101].

TIL and TKIs interaction with immune-cell infiltration

TIL correlated with poorer prognosis and shorter survival in RCC[102]. Liu *et al.* compared the percentage of immune cells in TKI-exposed RCC tissue with control samples and they found an increased CD3+ T-lymphocyte infiltration, CD45RO+ T-lymphocyte infiltration, CD4+ T-lymphocyte infiltration and CD8+ T-lymphocyte infiltration both after sunitinib and bevacizumab treatment. The infiltration of CD68+ macrophages was higher in sunitinib-treated versus control RCC specimens (25.2% *vs.* 20.3%, $P < 0.05$) but not in bevacizumab-treated samples (21.3% *vs.* 20.3%, $P > 0.05$). In sunitinib-treated patients, a higher percentage of tumor-infiltrating CD4+ T lymphocytes was associated with shorter OS and PFS. TIL composition might be modulated by VEGF target treatment. Indeed, although not changing either the ratio of CD4+FOXP3+ Tregs to total CD4+ T lymphocytes or the ratio of Treg to CD8+ T lymphocytes, CD4+FOXP3+ Tregs were greater in cases treated with bevacizumab or sunitinib.

Tregs infiltration was higher in sunitinib-treated patients with shorter OS and PFS. Finally, sunitinib directly enhanced PD-L1 expression, and those patients who had higher PD-L1 expression had shorter OS and PFS ($P < 0.05$) after receiving sunitinib treatment[103].

The analysis of T cell subsets and MDSCs in peripheral blood mononuclear cells (PBMCs) from ccRCC patients receiving cabozantinib and other therapies (nivolumab and pazopanib) revealed that T cell subsets composition changed after treatment. Indeed, cabozantinib treatment increased the proportion of Th9, Th22, and Th17 cells while having no effect on the number of Th2 cells, Th1, Treg, and CD8+ T cell populations. Among these T cells, the proportion of Th22, but not Th9, was associated with better outcomes[104].

Epigenetic modification

Non-coding and micro RNA

Non-coding RNAs, known as microRNAs (miRNAs), have significant functions in modulating the expression of genes. The role of miRNAs in resistance to TKIs is still being investigated[105].

Yamaguchi *et al.* conducted one of the first attempts to profile miRNAs in resistant RCC cell lines as they developed two sunitinib-resistant cell lines and performed microarray and RT-qPCR analysis on them. They identified seven miRNAs (miR-575, miR-642b-3p, miR-4430, miR-18a- 5p, miR-29b-1-5p, miR-431-3p, miR-4521) whose expression was linked to sunitinib resistance.

It was reported that miR-4430 had a role in modulating expression genes implicated in the inhibition of PTEN/mammalian target of rapamycin (mTOR) signaling pathway. miR-18a-5p is associated with hypoxia-inducible factor 1 alpha (HIF1A)[106]. Sunitinib- resistant cell lines had higher miR-4430 levels and lower miR-18a-5p ones, suggesting that the acquisition of sunitinib resistance was associated with PTEN downregulation, and FGF1 and HIF1A upregulation[107] [Table 3].

PRIMARY RESISTANCE TO ICIs

Availability of antigens and dendritic cells' (DCs') presentation of them, T-cell trafficking and tumor infiltration, T-cell effectiveness, and equilibrium between regulatory and cytotoxic cells in the TIME composition are all requirements for an immune response against tumor cells to be successful. Immune escape may occur in any of these phases, resulting in either primary or acquired resistance to immune checkpoint inhibitors[108].

Antigen availability and DCs presentation capacity

The recognition of a specific antigen by antigen-presentation cells (APCs) is the first step in a successful immune response. Major Histocompatibility Complex (MHC) proteins, which are classed as class I on all nucleated cells or class II on specific immune system cells such as macrophages, dendritic cells, and B cells, are also expressed on the surfaces of APCs. These proteins are necessary for the process of antigen presentation, which activates T cells in response to the antigen and results in a successful immune response[109].

Antigen availability may be influenced by the tumor mutational burden, and the absence of neoantigens may be caused by epigenetic alterations[110]. RCC has a relatively low mutational load. De Velasco *et al.* reported a low mutational load in their full exome transcriptome analysis of metastatic RCC patients included in TCGA, with a median of 1.42 mutations/Mb (range: 0.035-2.77). Classifying the 54 patients according to the IMDC risk criteria, no differences were seen in mutational load ($P = 0.39$), as well as in the

Table 3. Major determinants of primary and acquired resistance to VEGFR-TKIs

Primary resistance to VEGF-TKIs	
Missed targets	VHL-deficient tumors with HIF2 expression[40,41]
Insufficient intracellular drug concentration	MDR-related solute carrier (SLC) and ATB binding cassette (ABC)[44-46] Lysosomal sequestration[47-50]
Secondary resistance to VEGF-TKIs	
Tumor plasticity	Epithelial-mesenchymal transformation (EMT) induced by hypoxia and Insulin-growth factor (IGF)[54-64]
Non-angiogenic pathways	Vascular co-option
Bypass pathways	FGF/FGFR: FGF2-mediated activation of ERK 1/2 and PLC pathway is not inhibited by sunitinib[68] MET/HGF: Hypoxia induced by VEGFR-TKIs promotes MET-depended tumor growth[76-79]. GAS6/AXL: VEGFR-TKIs treatment enhances the activation of the MAPK pathway by AXL[90].
Tumor microenvironment interactions	Cytokines[91-93] ·IL-6 activates the AKT/mTOR pathway, inducing VEGFA and VEGFR2 expression. ·IL-8 induces VEGFA transcription even when HIF-1 is inhibited. MDSCs[100-102] ·GM-CSF confers sunitinib-resistance to peripheral MDSCs, via the STAT5 pathway. ·MDSCs produce pro-angiogenic proteins. CAFs[103] ·FAP mRNA (a marker of CAFs) levels are correlated with worse outcomes. TAMs[105-106] ·Hypoxia induces M2-macrophages phenotype. ·M2-like TAMs promote tumor growth. TILs[108] ·TKIs treatment induces CD4+ T cells and Tregs infiltration. ·CD4+ T cells and Tregs infiltration correlate with worse outcomes.
Epigenetic modifications	Non-coding RNAs and miRNAs[116] Sunitinib-resistant cells had higher miR-4430 (PTEN/mTOR signaling) and lower miR-18a-5p (FGF1 and HIF1A signaling) levels

CAFs: Cancer-associated fibroblasts; FAP: fibroblast associated protein; FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; HIF: hypoxia-inducible factor; HGF: hepatocyte growth factor; MDR: multidrug resistance; MAPK: mitogen-activated protein kinases; MDSC: myeloid-derived suppressor cells; miRNAs: microRNA; TAMs: tumor-associated macrophages; TILs: tumor-infiltrating lymphocytes; VHL: von hippel-lindau.

expression of cytolytic genes - granzyme A (GZMA) and perforin (PRF1) - or in selected immune checkpoint molecules (PD-1, PD-L1, PD-L2, CTLA-4) ($P > 0.05$ for all)[111].

Lack of antigen presentation could be affected by MHC mutations or defects in DCs' functions and maturation.

Beta-2-microglobulin (B2M), an important subunit of MHC class I, has essential biological functions and roles in tumor immunity. *B2M* gene deficiency and loss of Beta-2-microglobulin, induced by mutations and epigenetic regulation, can lead to complete loss of MHC class I antigen expression. B2M mutations frequently result in MHC defects that cannot be repaired, and immunotherapy is ineffective at restoring MHC expression[112].

The maturation of DCs can be influenced by numerous factors. Hypoxia, which creates an acidic environment with a higher amount of lactates, is one of these. Recent data suggests the existence of DCs with immune-suppressive characteristics. These cells may overexpress pathways involved in immune tolerance: e.g., STAT3 which induces S100A9, a gene that prevents DCs from maturing and recognizing antigens, or FOXO3, a transcription factor that induces the expression of indolamine 2,3-dioxygenase (IDO), arginase, and TGF-β and inhibits co-stimulatory molecules[113].

In the TIME, dendritic cells must already be present. A study conducted by Spranger *et al.* in melanoma murine models revealed that tumor-intrinsic active -catenin signaling results in T-cell exclusion and

resistance to ICIs. Tumors with active -catenin exhibited a nearly complete absence of activated T cells, according to an analysis of immunological infiltrates. This absence was attributed to the loss of dendritic cells, particularly CD103+ dendritic cells with diminished IFN-β expression. The number of CD103+ dendritic cells was similarly decreased in the tumor-draining lymph nodes, but not in the spleen, thus reflecting the fact that these DCs were not recruited due to a defect in chemotactic signals, such as CCL4. Furthermore, when treated with dual checkpoint inhibition, no therapeutic effect in reducing tumor growth was detected in active -catenin mice. Re-introduction of dendritic cells restored tumor response to ICI[114].

Human endogenous retroviruses

Human endogenous retroviruses (hERVs) are components of about 8% of our genome that possibly originated by incorporating ancestral exogenous viruses. There are only a limited number of tools available for quantifying and identifying hERVs. They could be a source of neoantigen and mediate immune response in TIME. Smith *et al.* identified more than 3,000 transcriptionally active hERVs within the TCGA pan-cancer RNA-Seq database. They explored two mechanisms by which hERV expression could affect the tumor immune microenvironment in ccRCC. The first mechanism involved innate immune response and activation of the RIG-I-like pathway signaling by double-stranded RNAs (dsRNA). The second was related to the adaptive immune response, as documented by the hHERV epitope-trigged activation of the B cells. Patients with both RIG-I–like downregulation and BCR-associated signatures upregulation had significantly shorter overall survival, while those with higher expression of the RIG-I-like signature had longer overall survival. Two significant tumor-specific hERVs were lastly found in ccRCC (CT-RCC hERV-E and hHERV 4700). The CT-RCC hERV-E displayed a Treg signature and was the second most differentially expressed hERV. In an aPD1-treated ccRCC dataset, hERV 4700 (HERVERI/gammaretro-virus-like) expression was higher in tumor samples compared to normal tissue, and it was associated with response to immunotherapy. This could occur because hERV 4700-derived epitopes may be the target of an antitumor response mediated by aPD1 mAb[115,116].

Immune cell trafficking and recruitment

As discussed above, activation of the WNT/-catenin pathway reduces the recruitment of dendritic cells in TIME, affecting T-cell activation[114].

Given that pre-existing T-cells in the tumor are essential for response to ICIs, various mechanisms that may cause initial resistance to immunotherapy with the end result of the absence of immunologically competent cells in TIME have been investigated so far.

PTEN is a lipid phosphatase that inhibits the activity of PI3K/AKT signaling. PTEN loss has been associated with altered TIME T-cell composition. Peng *et al.* found that a lower CD8+ T cell tumor infiltration was present in melanomas with PTEN loss compared to tumors with PTEN expression ($P < 0.001$). Furthermore, PTEN loss resulted in both an up-regulation of inhibitory cytokines including CCL2 and VEGF and a down-regulation of the T cell effector molecules IFN- and granzyme B. Since PTEN loss and PI3K/AKT pathway signaling both contribute to immunotherapy resistance, it is possible to reverse this effect by targeting the PI3K-AKT pathway[117].

T-cell activity in TIME and microenvironment composition

T cells get activated when they come into contact with APCs and the MHC complex through the TCR. Following this, T cells mostly develop into cytotoxic T cells (CD8+ cells) or T-helper (Th) cells (CD4+ cells), and they release cytokines that further regulate the immune response[108]. Cytotoxic CD8+ T lymphocytes are mainly responsible for killing tumor cells. MHC class II proteins trigger the activation of CD4+ Th cells, which lack the ability to cause cytotoxicity. In fact, they are implicated in the recruitment of additional

immune cells, producing cytokines. IFN-γ is a mediator of the Th1 cell response[109].

As tumors grow, persistent exposure to IFN-γmay be responsible for a down-regulation of IFN signaling downstream, resulting in primary immunotherapy resistance. Mutations in IFN receptors, Janus kinases JAK 1/JAK2, are studied mechanisms by which cancer cells could have an advantage in developing resistance to IFN-mediate anti-proliferative effects. Shing *et al*. discovered that loss of function mutations in JAK1/2 in melanoma cells could inhibit T cells killing capacity and IFN's ability to induce PD-L1 expression, making pharmacological suppression of the PD-L1/PD-1 interaction ineffective. Reduced T-cell trafficking, along with decreased production of chemokines such as CXCL9, CXCL10, and CXCL11, could potentially account for the lack of response[118].

For a competent immune response to be effective, there must be a balance between cells that inhibit immunity and cells that promote response inside the tumor microenvironment. TME is dynamic and constantly changes. The TIME of ccRCC is unusual, and it is distinguished by a strong inflammatory profile and T-cell infiltration. The primary immunosuppressive cells in TIME are TAMs, MDSCs, and regulatory T cells (Treg)[119].

TAMs

In ccRCC, cancer-cell-derived factors such as IL-1β, IL-6, IL-10, tumor necrosis factor-α, epidermal growth factor, and TGF-β induce macrophage polarization towards a M2 phenotype by cell-cell interactions. CD163+ M2 TAMs contributed to poor clinical prognosis in patients with ccRCC by activating STAT3 pathway. PI(3)Kinase γ (PI3Kγ) is found to be implicated in themacrophage switch from a M1-phenotype to a M2-phenotype, inhibiting Akt and mTOR together with the NFκB and C/EBPβ activation. As a result, the immune suppressive phenotype promotes tumor growth and inflammation[120,121].

Tregs

Through the production of IL-10 and TGF-, Tregs can mediate immunosuppression by inhibiting T cells and APCs functions. These cells can be recruited in TIME by chemokines and cytokine produced by exhausted T-cells. Furthermore, cancer cells can play out escape mechanisms upregulating Tregs in TIME[109]. Hyperactivation of focal adhesion kinase (FAK) (also known as FADK) in tumor cells induces overexpression of various chemokines (including CCL5), thus recruiting Treg cells and inducing CD8+ T cell exclusion or exhaustion. Self-antigen-specific CD8+ T cells express restricted co-stimulatory signals, which impair APC activity and enhance suppression by Tregs, whereas Tregs have a stronger affinity for TCRs of non-self-antigen specific CD8+ T cells, which are resistant to suppression[121,122].

Immune checkpoints like CTLA-4 and PD-1 are expressed by Tregs as well. In human glioblastoma tissue, Tregs with high PD-1 exhibit an exhausted phenotype lacking immunosuppressive activity. Anti-PD-1 mAbs may cause hyper-progression, decreasing the number of Tregs that express PD-1 and restore their ability to inhibit immunological response[121,123-125].

The contribution of the B7x immunological checkpoint to the growth of the Treg population within the tumor was discussed by John *et al*. In accordance with their suppressive function, B7x+ Tregs exhibited a higher level of TGF-LAP (a surface marker of TGF production) and a lower percentage of ki67 marker, indicating that they originated from peripherally converted CD4+ T cells. B7x increased Foxp3 expression through increasing STAT5 phosphorylation, whereas it inhibited STAT3 phosphorylation, which affected CD4+ T cells' ability to differentiate into the Th17 subtype. What is more intriguing is that the anti-CTLA-4 therapy was not successful in reducing Tregs exclusively in mice that expressed B7x+. In addition, B7x+

mice showed a substantial reduction in the IFN production generally caused by anti-CTLA-4. Tregs play a crucial part in the immune response, as shown by the fact that Treg depletion restored anti-CTLA-4 capabilities in tumor reduction even in B7x+ mice[126].

MDSCs

MDSCs may differentiate into M2-polarized macrophages with immunosuppressive properties and can decrease T-cell activation by metabolic processes (e.g., iNOS, IDO). Additionally, they are able to express CD40 and to produce TGF- and IL-1, leading to Tregs expansion and decreasing the capacity of effector T cells[93]. In both melanoma patients and prostate cancer patients treated with ipilimumab, a lower baseline amount of circulating MDSCs was associated with a higher overall survival rate. Furthermore, high myeloid inflammation gene signature expression was associated with reduced PFS in the atezolizumab monotherapy arm and in the atezolizumab + bevacizumab arm, but not in the sunitinib arm in the molecular analysis of the IMmotion 150[127,22].

DCs

According to their presence in Tertiary lymphoid structures (TLS), DCs in ccRCC can be divided into two subtypes: TLS-DCs (CD83+ DC- LAMP+), which are very uncommon in ccRCC and associated with good outcomes, and non-TLS-DCs (CD209+ CD83), which are dominant in ccRCC TIME and associated with the worst prognosis. In addition to promoting tumor growth by secreting MMP-9 and TNF, NTLS-DCs further inhibit CD8+ T-cell activity by the L-arginine pathway and trigger Treg responses by secreting TGF-[109,119,128,129].

Genomic and single-gene mutation and TIME

The biomarker analyses conducted among patients treated in the IMmotion 150, the Javelin Renal 101 and the Checkmate 214 had developed the idea that gene expression profile (GEP) in RCC could predict response to ICIs[22-24].

Based on mRNA expression data, Beuselinck *et al.* performed a clustering analysis and identified four molecular subgroups of ccRCC, even if done in a cohort of patients receiving sunitinib: ccrcc1 ("c-myc-up"), ccrcc2 tumors ("classical"), ccrcc3 ("normal-like") and ccrcc4 tumors ("c-myc-up and immune-up"). The ccRCC4 subtype displayed a strong inflammatory, Th1-oriented but suppressive immune microenvironment, with a strong expression of myeloid and T-cell homing factors. Furthermore, this subtype had a higher proportion of IL10 as well as inhibitory receptors LAG3 and PD-1 and PD-1 ligands PD-L1 and PD-L2. In the validation analysis of single-gene mutations conducted on TGCA samples, SETD2 mutations were related to a lower T-cell infiltration and immunosuppressive markers (ccrcc1), while BAP1 mutations were expressed in the subtype with the highest inflammatory infiltration but on the other hand, having the strongest expression of immunosuppressive cells (ccrcc4)[130].

In the BIONIKK trial, the authors used Beuselinck's clustering classification to undertake a biomarker-driven analysis. Patients with the ccrcc 1 and 4 subtypes were randomized to receive nivolumab either alone or in combination with ipilimumab, whereas those with ccrcc 2 and 3 could receive either nivolumab plus ipilimumab or a VEGF-TKI. In the ccrcc1 (immune desert subtype), ORR and PFS were improved by the dual checkpoint inhibition (HR of PFS for nivolumab *vs.* nivolumab + ipilimumab 1.27; 95%CI 0.77-2.11). In the ccrcc4 (immune infiltrated and inflammatory subtype), both nivolumab alone and in combination with ipilimumab obtained higher ORR and longer PFS compared to the ccrcc 1 group. Thus, ccrcc4 seemed the best candidate for dual checkpoint inhibition. Furthermore, about 30% of patients in the ccrcc4 group who early progressed on nivolumab–ipilimumab did not start a second-line therapy, thus reflecting that

progression at first evaluation, does not always indicate resistance[131].

PBRM1 and BAP1

PBRM1 encodes for BAF180, a component of the SWI/SNF chromatin remodeling complex and could be inactivated in about 36% of clear cell renal cell carcinoma. Its relationship with prognosis in RCC has been evaluated in several studies, resulting in conflicting findings. PBRM1 mutations have also been documented in VHL-disease-associated RCC. PBRM1 and VHL mutations were most frequently expressed in ccrcc1 subtypes (immune desert one)[131-134]. In the phase I CA209-009, 35 samples of RCC treated with Nivolumab were whole-exome sequenced and were consequently divided into three categories according to responses. Clinical response to Nivolumab was characterized by a higher percentage of PBRM1 loss-of-function. The same results were seen in a subgroup treated with nivolumab plus ipilimumab[135].

As reported above, in the molecular subsets analysis of the IMmotion 151, PBRM1 mutations conferred better outcomes to patients, regardless of the treatment arm. However, in patients with PBRM1-non-mutant tumors, the addiction of immunotherapy to a VEGFR-TKI confers better outcomes compared to target therapy alone[31].

SETD2

SETD2 mutations, a histone methyltransferase gene, occur in 10% of ccRCC. Wang *et al.* clustered TGCA RCC samples based on TME expression profiles and observed two different clusters: the first cluster, inflamed subtype (IS), was enriched for Treg cells, NK cells, Th cells, neutrophils, macrophages, eosinophils, B cells and CD8+ T cells, whereas the second cluster, not inflamed subtype (NIS), was enriched for angiogenesis, plasmacytoid DCs, and mast cells. Mutations in BAP1 were most frequently seen in the IS ($P = 7.7 \times 10^{-5}$), whereas the NIS was enriched for PBRM1 mutations. Furthermore, the authors observed that Bap1-mutated mice were more infiltrated by CD4 and CD8 T cells, than Pbrm1-mutated mice, suggesting a causal relationship between BAP1 mutations and the TME-IS phenotype. It is interesting to note that individuals with tumors of the TME-IS subtype had higher rates of thrombocytosis and anemia, which are systemic symptoms of inflammation caused in TME[136].

GUT MICROBIOME INFLUENCES PRIMARY RESISTANCE TO IMMUNOTHERAPY

The complex system of the gut microbiota interacts with the host. By producing neoantigens and modifying the tumor immunological microenvironment, microorganisms have an impact on immune responses. Furthermore, studies have revealed that antibiotics can affect the metabolic balance of the intestinal microbiome by increasing some species while decreasing others, leading to dysbiosis[137-140].

Routy *et al.* demonstrated that the use of antibiotics (ATB) influenced response to ICIs in non-small cell lung cancer (NSCLC), RCC and urothelial cancers. As evidenced by poorer outcomes for individuals receiving antibiotic treatment, ATB usage did, in fact, cause resistance to PD-1 blockade in all tumor types. Analyzing the microbiome composition, *Akkermansia muciniphila* conferred better PFS to patients treated with immunotherapy. Th1 and Tc1 cell reactivity against *A. muciniphila* was the only immune response to ICIs that was associated with clinical outcomes[138].

De rosa *et al.* confirmed that ATB impaired patients' responses to immunotherapy by altering the diversity and composition of gut microbiota in a metagenomic and network analysis of patients treated with nivolumab in the NIVOREN GETUG-AFU 26 phase 2 trial. Responders had an over-representation of distinct species including *A. muciniphila, Bacteroides salyersiae*, and *Eubacterium siraeum*, and a trend towards *Clostridium ramosum* and *Alistipes senegalensis*, whereas *E. bacterium*_2_2_44A, *Clostridium*

hathewayi, and *Clostridium clostridioforme* were more represented in non-responders, as observed for those patients using ATB. Due to the fact that the majority of patients had already received a VEGF-TKI, the authors examined the effects of TKI use in combination with ATB on microbiome composition and discovered that axitinib + ATB was the most effective treatment to induce a shift in fecal microbiota. Additionally, most notably with cabozantinib, TKIs promoted a transition to a preponderance of immunostimulatory commensals, such as A. senegalensis and A. muciniphila. This property could be one of the rational bases for combining VEGFR-TKI with ICIs[141].

ACQUIRED RESISTANCE TO IMMUNE CHECKPOINT INHIBITORS

Secondary resistance develops throughout therapy and may be driven by the stress that a particular therapy might have on TIME, as well as by temporal variability and plasticity. The same mechanisms that lead to initial resistance also cause secondary resistance to immune-checkpoint inhibitors. Some investigators observed that TILs (the immune response's effectors) are present during relapse but remain restricted to the tumor margin, raising the possibility that these cells have lost the ability to identify antigens or to activate themselves. Utilizing ICIs, the IFN-induced expression of PD-L1 and its negative effects on CD8+ T cells are prevented. To reduce antigen presentation or enable escape from interferon-induced growth inhibition, cancer cells may, however, become insensitive to IFN signaling. JAK mutations have been investigated as a strategy by which cancers develop IFN unresponsiveness[142].

Numerous investigations conducted on humans have shown that tumor cells can develop resistance to T-cell recognition by having defective HLA class I expression[142-145]. Loss-of-function in chromosome region 15q21-(where β2m gene maps) induces a *β2m* gene mutation, leading to the absence of the MHC class I. These alterations can be reversible and the HLA expression can be recovered by using immunotherapy, as a result of transcriptional silencing of genes or irreversible. It has been suggested that resistance to immunotherapy may be caused by the preexistence of metastatic lesions with a *β2m* gene mutation and that selective pressure during T-cell-based immunotherapy could induce the growth of HLA class-I deficient clones in melanoma patients who have irreversible HLA alterations[146].

Chronically activated Treg cells showed strong suppressive potential. Tregs increase CD103 expression when activated, and CD103+ Treg exhibited greater levels of inhibitory receptors such as PD-1, TIM-3, and CTLA-4. Additionally, they upregulate functional molecules, granzyme B and IL-10, which are essential to their suppressive actions, such as the decrease in CD8+ T cells[147].

ALTERNATIVE INHIBITORY RECEPTORS UPREGULATION AS A MECHANISM OF ACQUIRED RESISTANCE

Exhausted CD8+ T cells lose their cytotoxic efficacy against presented antigens. PD-1 is only one of the inhibitory receptors highly expressed on exhausted CD8 T cells and its blockade may have a therapeutical effect. However, blocking this pathway does not completely restore T cell function. Numerous alternative inhibitory receptors, primarily identified in chronic infections, such as LAG-3, TIM-3, 2B4, CD160, or BTLA, are overexpressed in exhausted T cells and may be targeted to enhance immune response or reverse resistance[147,148].

PD-1

PD-1 is an inhibitor of both adaptive and innate immune responses, and it is expressed on activated T, natural killer (NK) and B lymphocytes, dendritic cells (DCs), macrophages, and monocytes[149]. In a cohort of patients with NSCLC, gastric cancer (GC) and melanoma treated with Nivolumab, CD8+ T cells of the TIMEs from responders expressed higher levels of PD-1 than those from non-responders. Furthermore,

PD-1 was highly expressed in eTreg (CD45RA–CD25^{hi}Foxp3^{hi}CD4+) of the TIME of patients with NSCLC and GC which not responded to nivolumab. In this context, the use of aPD-1 mAb could have immunosuppressive effects, enhancing the expression of PD-1 among eTreg, and thus stimulating Treg functions[150].

PD-1 expression balance between CD8+ T cells and Treg cells is crucial for the efficacy of monoclonal antibodies to PD-1.

The prognostic value of PD-L1 expression in RCC is controversial. A meta-analysis of four trials assessed the predictive value of PD-L1 among patients treated with nivolumab + ipilimumab, atezolizumab + bevacizumab, pembrolizumab + axitinib or avelumab + axitinib *vs.* sunitinib. Patients with PD-L1-positive tumors showed significantly improved ORRs, complete response rates (CRRs) and PFS, and lower progression disease responses (PDRs) compared with those with PD-L1-negative tumors when treated with ICIs *vs.* sunitinib. Nivolumab plus ipilimumab had the highest likelihood of providing the maximal PFS (p score: 0.90) and the highest ORR (p score: 0.95)[151].

Overall, even though the findings of this study show that PD-L1 has a predictive value, there are several limitations, such as the fact that PD-L1 is expressed differently in primary tumors compared to metastatic sites, as well as the lack of a standard detection method and the different types of anti-PD-L1 antibody used. Examining the variable pattern of PD-L1 expression on different immune cells in TIME might be more fascinating than examining its expression just on tumor cells. As reported, it appears that the effectiveness of ICIs depends on the balance between PD-1 expression on CD8+ T-cells and Treg.

LAG3

Lymphocyte Activation Gene-3 (LAG3; CD223) is expressed on activated human NK and T cell lines and is able to bind MHC class II. A soluble monomeric form of LAG3 (sLAG3) can be released by IFN-producing CD4+ T cells. The major ligand of LAG3 is MHC class II. Melanoma cells that express MHC class II attract tumor-specific CD4+ T cells through their interaction with LAG3, resulting in impairing CD8+ T cell responses. Other putative ligands are Galectin-3, which mediates suppression of CD8+ T-cell-secreted IFN in vitro and LSECtin (liver sinusoidal endothelial cell lectin). It was found that the association between LAG3 and the LSECtin ligand inhibits the generation of IFN by effector T cells that are antigen-specific in melanoma cells. Dendritic cell inhibition is one of the ways whereby LAG3-expressing Tregs interact with MHC class II to cause immunological suppression. In fact, when exposed to LAG-3, MHC class II-expressing melanoma cells, but not MHC class II-negative ones, were resistant to Fas-mediated apoptosis[152-155].

In a study examining the key inhibitory receptors (iR) expression on TILs and PBMCs of 35 patients with RCC, CD8+ T cells and non-Treg CD4+ cells highly expressed the inhibitory receptors PD-1, followed by LAG-3 and BTLA, whereas Tim-3 and CTLA-4 were less highly expressed. However, the expression profile of the five iR on tumor-infiltrating Tregs was different. Indeed, Tregs upregulated PD-1, LAG-3, Tim-3, and CTLA-4, but not BTLA. Interestingly, as previously reported, the most frequent iR combination was PD-1 and LAG-3, whereas about 10% of CD8+ T cells expressed PD-1, LAG-3 and Tim-3 simultaneously. Experimental in vitro demonstrated that blockade of PD-1 plus LAG-3 resulted in a statistically significant higher percentage of CD8+ IFN+ T cells, than blockade of PD-1 alone.

Additionally, when CD4+ and CD8+ T cells were co-cultured with anti-PD-1, LAG3 upregulated but not PD-1. These findings suggested that blocking LAG-3 in combination with PD-1 might be an effective

treatment for advanced RCC[156].

TIM-3

T cell immunoglobulin and mucin-domain containing-3 (Tim-3) is a type I trans membrane protein expressed in IFN-γ-producing Th1 and Tc1 cells. The expression of cytokines such as TNF and INF-γ and Th1 responses are significantly suppressed by Tim-3. Tim-3 is linked to T cell exhaustion and its expression on CD8+ T cells is directly related to PD-1. Indeed, CD8+ T cells co-expressing Tim-3 and PD-1 are "deeply" exhausted T cells. Tim-3 could be expressed on tumor-infiltrating DCs, playing a role as a mediator of the innate immune response. The Tim-3 ligand with the highest affinity is galectin-9, and its interaction with Tim-3 causes the death of effector Th1 cells and CD8+ T cells. Furthermore, galectin-9 increases Tim-3-mediated IFN production in NK cells, activates PI3K-mTOR signaling in myeloid cells, and alters cytokine production by monocytes/macrophages, affecting Th1 and Th17 responses.

Other theorized ligands are being researched. Galectin-9 and Ceacam1 collaborate, both having a comparable effect. Activated DCs release the damage-associated molecular pattern known as HMGB1 (high-mobility group box 1), which stimulates T and B cell responses while inhibiting innate immune responses to tumor-derived nucleic acids. Tim-3 signaling's impact is influenced by the ligands involved, the cellular environment, and the biological state, particularly whether the stimulation is acute or persistent. Tim-3 can have adjuvant effects inducing the expression of co-stimulatory receptors. In contrast, chronic stimulation could enhance the inhibitory functions of Tim-3 signaling, especially as concerns HLA-B associated transcript 3 (Bat3) deficient Th1 and CD8+ T cells, driving these cells to an exhaustion stage[157-164].

Expression of Tim-3 on T cells also plays a critical role in the generation of MDSCs and is even present in FoxP3+ T regs, contributing to promoting T cell dysfunction and immune suppression[165,166].

In isolated CD8+ T cells, Tim-3+PD-1+ TILs were identified as an exhausted phenotype of T cells, having a reduced production of IL-2, TNF, and IFN-γ. Combining anti-Tim-3 and anti-PD-L1 therapy reduced the tumor growth in mouse models, and those who underwent a complete regression continued to be tumor-free even after rechallenging[167].

Tim-3 expression in RCC has been associated with outcomes resulting in contradictory results. Patients receiving nivolumab were assessed in the CheckMate-010 research study conducted by Pignon *et al.* in anattempt to identify the mechanisms causing different responses. Longer median immune-related response progression-free survival (irPFS) and higher ORR were associated with the presence of CD8+ tumor-infiltrating cells that express PD-1 but lack LAG3 and TIM3 and are more likely to be T cell activated. This is in contrast to Zelba *et al.*'s findings, which showed that blocking both PD-1 and Tim-3 simultaneously had no effect on the average cytokine production by TILs, resulting in a lack of the immune response's restoration. Therefore, additional research is required to confirm the usefulness of Tim-3 as a possible target to enhance immune responses driven by the inhibition of checkpoint inhibitors[168,156] [Table 4].

CONCLUSIONS

Metastatic renal cell carcinoma management has undergone a paradigm shift as a result of the development of combination therapy using ICIs. The choice of first-line treatment and its correct application continues to be crucial factors in tumor evolution and have the potential to cause initial resistance, which may have an impact on overall survival. Because of this, it is now crucial to understand how the combination of ICIs or the addition of a VEGF-TKI to immunotherapy may alter the tumor microenvironment and affect the

Table 4. Major determinants of primary and acquired resistance to ICIs

Primary resistance to ICIs	
Antigen availability	mRCC has a low mutation load
Antigen presentation defects	Beta-2 microglobulin loss might result in a deficiency of MHC class I expression.
Absence of dendritic cells (DCs)	Due to defects in chemotactic signals, active -catenin signaling causes the depletion of DCs.
Antigen epigenetic modification	hHERVs may influence both innate and adaptative immune responses.
Cells trafficking and recruitment alterations	·WNT/-catenin pathway activation reduces DCs recruitment. ·PTEN loss reduces CD8+T cell infiltration.
T-cell activity inhibition	JAK1/2 loss of function mutations inhibit T cell killing efficacy and IFN-mediated PD-L1 expression.
Tumor microenvironment composition	TAMs ·M2-like TAMs correlate with worse prognosis in mRCC Tregs ·Anti-CTLA-4 mAbs are not effective in depleting Tregs expressing B7x immune checkpoint. MDSCs ·Low MDSCs infiltration correlates with better outcomes with ICIs DCs ·A specific subtype of DCs (NTLS-DCs) with immunosuppressive functions is dominant in ccRCC.
Genomic and single-gene mutations	PBRM1 ·Regardless of the type of treatment utilized, PBRM1 mutations are correlated with better results. ·Combining VEGFR-TKI with ICI is more effective in PBRM1 non-mutant tumors. ·PBRM1 mutations are more frequent in non-inflamed, angiogenic subtypes. SETD2 ·SETD2 mutations are enriched in inflamed immune infiltrated subtypes.
Gut microbiome composition	Antibiotic use induces resistance to aPD-L1 mAb Different bacterial species are represented in responders *vs.* non-responders
Secondary resistance to ICIs	
Same mechanisms involved in primary resistance	·IFN unresponsiveness via JAK mutations ·HLA I defects. ·Loss-of-function of Beta-2 microglobulin mutations.
Alternative inhibitory receptors	·LAG3 ·TIM-3

DCs: Dendritic cells; hHERVs: human endogenous retroviruses; HLA: human leukocyte antigen; IFN, interferon; JAK: janus kinases; LAG3: lymphocyte activation gene-3; MDSCs: myeloid-derived suppressor cells; mRCC: metastatic renal cell carcinoma; PD-L1: programmed death-ligand 1; TAMs: tumor-associated macrophages; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; Tregs: T regulator.

tumor's response to treatment. Targeting hypoxia with specific drugs may be a possibility to enhance outcomes in RCC since hypoxia is a defining feature of RCC pathogenesis and is associated with both angiogenesis and the alteration of the tumor microenvironment. HIF-2 inhibitor belzutifan is being tested in patients with previously treated mRCC as a single treatment versus everolimus (NCT04195750) or in combination with lenvatinib versus cabozantinib (NCT04586231). Different inhibitory receptors or metabolic pathways may be the focus of alternative approaches. The phase II trial FRACTION-RCC is studying an anti-LAG3 mAb (relatlimab), in combination with nivolumab (NCT02996110). XmAb®22,841, a bispecific antibody targeting CTLA-4 and LAG-3, is under study in the phase I trial DUET-4 (NCT03849469). A randomized phase II trial is investigating the efficacy of axitinib combined with an antibody against OX40, a receptor expressed on memory T cells (NCT03092856). In a phase 1b/2 trial, patients with previously treated mRCC are being treated with cabozantinib in conjunction with the anti-AXL fusion protein AVB-S6-500, which controls the GAS6/AXL signaling pathway (NCT04300140).

Despite the previously mentioned advancements, there is still a significant research gap in the individual biology of tumors. Furthermore, there is no trustworthy biomarker to direct patient selection. The dynamic genomic and immunomodulatory alterations that systemic treatment causes in the TIME in advanced ccRCC may help to partially explain this paucity of biomarkers.

It may be possible to predict therapy response by combining tumor genomic and immune signatures, but much more accurate research is needed to link biological understanding with clinical findings in RCC.

We must change how we approach treating ccRCC due to the advent of clinical resistance to the currently available systemic treatments. Cancer cells are constantly changing as a result of therapy pressure, plasticity, and heterogeneity. We may be able to comprehend, avoid, and overcome resistance mechanisms by estimating the trajectory of ccRCC evolution. In the prospective cohort study TRACERx, the authors conducted a whole genome sequencing of RCC tumor samples to generate information on the timing of driver mutations, level of intratumoral heterogeneity, and presence of parallel evolution in each patient. They found that the loss of heterozygosis of chromosome 3p was the first critical driving event. The most frequent alteration was rearrangement between 3p and 5q (one copy of 3p lost and one copy of 5q earned), defined t (3:5) chromothripsis. Using t (3:5) as the cut-off, they calculated the chronological age at which each mutation occurred. They found that the duplication that caused t (3:5) chromothripsis was an early event (35-50 years before tumor diagnosis), causing a modest initial clonal expansion, and that the mutation rate throughout life remained constant. Due to the latency between the triggering mutational event and subsequent progression, there may be a window for early intervention to prevent RCC. Indeed, the incidence of sporadic RCC could be decreased by reducing the 3p-LOH clone size by 50%. This is reasonable given that this chromosome contains four tumor-suppressor genes (VHL, PBRM1, BAP1, and SETD2) that are crucial for cellular survival[169].

Based on seven evolutionary subgroups that coincide with clinical characteristics, ccRCCs have been divided into four groups[169,170]. These groups are distinguished by four features-variations in chromosomal complexity, intra-tumor heterogeneity (ITH), model of tumor evolution, and metastatic potential.

In a review by Kowalewski *et al.*, the authors investigate single group characteristics and describe possible evolution-target strategies according to the evolutionary trajectories. Group 1 tumors are those that have a single VHL mutation and a low genome instability index (wGII), as well as low ITH. Given the positive predictive significance of a low wGII as a measure of response to ICIs, this group may benefit from immunotherapy. Furthermore, a stable tumor burden could be reinforced by adaptative therapy, upfront cytoreductive nephrectomy (CN), and treatment targeting trunk group before the loss of 9p or 14q, which marks the acquisition of metastatic competence. Group 2 tumors are those with an early PBRM1 mutation and a subsequent SETD2 mutation, PI3K pathway mutation, or high wGII, and were distinguished by a "branched" evolutionary pattern. In this group, modulating genomic instability could be useless, whereas targeting immune evasion could be an option. In contrast, Group 3 and 4 tumors are those with multiple driver mutations (VHL plus ≥ 2 BAP1, PBRM1, SETD2, or PTEN) resulting in "punctuated" evolution and characterized by high wGII. ITH is low in group 3 tumors but higher in group 4 tumors, giving that group a rapid dissemination pattern. Due to high wGII and a punctuated evolution pattern in Groups 3 and 4, it may be effective to address genomic instability by enhancing it. The goal of evolutionary herding is to reduce ITH and manage any potential distinct clones that may result from a prior treatment by utilizing a combination of drugs in a specific order. Hence, it should be considered in Groups 1 and 3[171].

We might be able to transpose this perspective into the real world with the aid of new technologies. For instance, the repeated evolution in cancer (REVOLVER) machine-learning algorithm was created to achieve repeatable disease prognosis based on next-generation sequencing (NGS) count data, thereby classifying patients based on the evolution of their tumors over time[172]. Trials including biomarkers and evolutionary paths as drivers of chosen treatment will be the new challenge in the future, in order to predict earlier the correct strategy and to prevent a manipulation that could be harmful when applied in the incorrect evolutionary trajectory.

DECLARATIONS

Authors' contributions

Conceptualization, formal analysis, resources, data curation, writing - original draft: Astore S
Data curation, resources, software, writing - review and editing: Baciarello G, Cerbone L
Validation, supervision, project administration, writing - review, project administration: Calabrò F

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SA declares no conflicts of interest. FA is a consultant/member of the external advisory board for Pfizer, BMS, Ipsen, MSD, AstraZeneca, Merck and Accord.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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A comprehensive overview of recent developments on the mechanisms and pathways of ferroptosis in cancer: the potential implications for therapeutic strategies in ovarian cancer

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Abstract

Cancer cells adapt to environmental changes and alter their metabolic pathways to promote survival and proliferation. Metabolic reprogramming not only allows tumor cells to maintain a reduction-oxidation balance by rewiring resources for survival, but also causes nutrient addiction or metabolic vulnerability. Ferroptosis is a form of regulated cell death characterized by the iron-dependent accumulation of lipid peroxides. Excess iron in ovarian cancer amplifies free oxidative radicals and drives the Fenton reaction, thereby inducing ferroptosis. However, ovarian cancer is characterized by ferroptosis resistance. Therefore, the induction of ferroptosis is an exciting new targeted therapy for ovarian cancer. In this review, potential metabolic pathways targeting ferroptosis were summarized to promote anticancer effects, and current knowledge and future perspectives on ferroptosis for ovarian cancer therapy were discussed. Two therapeutic strategies were highlighted in this review: directly inducing the ferroptosis pathway and targeting metabolic vulnerabilities that affect ferroptosis. The overexpression of SLC7A11, a cystine/glutamate antiporter SLC7A11 (also known as xCT), is involved in the suppression of ferroptosis. xCT inhibition by ferroptosis inducers (e.g., erastin) can promote cell death when carbon as an energy

source of glucose, glutamine, or fatty acids is abundant. On the contrary, xCT regulation has been reported to be highly dependent on the metabolic vulnerability. Drugs that target intrinsic metabolic vulnerabilities (e.g., GLUT1 inhibitors, PDK4 inhibitors, or glutaminase inhibitors) predispose cancer cells to death, which is triggered by decreased nicotinamide adenine dinucleotide phosphate generation or increased reactive oxygen species accumulation. Therefore, therapeutic approaches that either directly inhibit the xCT pathway or target metabolic vulnerabilities may be effective in overcoming ferroptosis resistance. Real-time monitoring of changes in metabolic pathways may aid in selecting personalized treatment modalities. Despite the rapid development of ferroptosis-inducing agents, therapeutic strategies targeting metabolic vulnerability remain in their infancy. Thus, further studies must be conducted to comprehensively understand the precise mechanism linking metabolic rewiring with ferroptosis.

Keywords: Ferroptosis, glutaminolysis, glycolysis, metabolic vulnerability, ovarian cancer, pentose phosphate pathway

INTRODUCTION

Epithelial ovarian cancer has a poor prognosis because of limited early screening methods and high recurrence rates[1]. Modern approaches include debulking surgery, platinum/taxane chemotherapy, angiogenesis inhibitors (e.g., bevacizumab), drugs targeting poly(ADP-ribose) polymerase (i.e., PARP inhibitors), and drugs targeting the dysfunctional immune system (i.e., immune checkpoint inhibitors) depending on the histological type and tumor stage[2]. Thus, establishing more effective treatment options is necessary as ovarian cancer often recurs and becomes resistant to chemotherapy.

High-grade serous ovarian cancer (HGSC) is a common and aggressive subtype of epithelial ovarian cancer[3]. Clear cell carcinoma (CCC) of the ovary, the second most common histological type in Japan, has distinctive clinical behavior, biological function, and molecular characteristics[3]. HGSC arises from the implantation of fallopian tube epithelial cells, and it is characterized by TP53 (p53) mutations associated with enhanced genomic instability[3]. p53 mutation may promote hypoxia-induced genomic instability, leading to the activation of pro-oncogenic signaling such as hypoxia-inducible factor (HIF) 1[4]. Furthermore, fallopian tube epithelial cells are constantly exposed to potentially toxic constituents [e.g., labile iron and reactive oxygen species (ROS)] derived from retrograde menstrual reflux even before the accumulation of somatic or oncogenic mutations[5]. The iron content determined in HGSC was almost five times higher than that for normal ovarian cells[6]. HGSC exhibits an increased expression level of the iron import transferrin receptor 1 and a decreased expression level of the cellular iron exporter ferroportin, thereby enhancing the intracellular iron pool[7]. In addition, based on previous reports, CCC derived from endometriosis could increase CCC cell proliferation via iron supplied by the surrounding endometriosis[8]. Rapidly proliferating cancer cells have a unique phenotype of iron metabolism, which increases iron supply and decreases iron loss[9]. Apart from iron, such cancer cells also require a continuous supply of adequate nutrients and oxygen available within the tumor microenvironment to generate their own bioenergetics (e.g., glucose, glutamine, cysteine, ATP, and fatty acid) and macromolecules (e.g., proteins, lipids, and nucleic acids)[10]. Glycolysis, mitochondrial oxidative phosphorylation (OXPHOS), glutaminolysis, and fatty acid synthesis are the major pathways of energy metabolism. Ovarian cancer cells switch from OXPHOS to aerobic glycolysis to adapt to environmental changes[11] through HIF-mediated metabolic reprogramming[12].

Cancer cells acquire a diverse range of metabolic flexibility and plasticity as an adaptation to ever-changing nutritional microenvironments during tumor evolution[13]. However, accelerated energy metabolism that supports the enhanced proliferation rate further increases iron demand, oxidative stress, and susceptibility to tumor cell death[14]. Ferroptosis is a recognized form of regulated cell death associated with iron and ROS

accumulation, which serves as a vital component of various processes in ovarian cancer[7,15]. Under nutrient-deprived conditions, cancer cells depend on optimal metabolic pathways for survival. This metabolic reprogramming creates an addiction to intracellular and extracellular nutrients (i.e., strong dependencies on nutrients), which may result in acquired resistance to cell death[13]. Metabolic flexibility and nutrient addiction are critical for cell fate determination. Therefore, forced alterations in metabolic pathways may have great application potential in tumor-targeted therapy. Thus, this review aims to summarize the regulation of ferroptosis evasion and its crosstalk with multiple cellular metabolic pathways in a variety of cancers and to discuss research perspectives, particularly therapeutic strategies targeting ovarian cancer.

METABOLIC FLEXIBILITY, PLASTICITY, AND VULNERABILITY IN CANCER CELLS

Cells have evolved elaborate metabolic flexibility to control cell survival, growth, and defense against oxidative stress for adaptation to ever-changing aerobic or anaerobic environments[16]. Glucose is the major energy source for eukaryotic cells, and it plays a critical role in redox (reduction/oxidation) homeostasis[10]. Glucose is transported into cells via transmembrane proteins [e.g., glucose transporter (GLUT)] and metabolized through glycolysis and the pentose phosphate pathway (PPP), an early diverging branch of glycolysis[17]. The internalized glucose molecule is initially converted to glucose 6-phosphate (G6P) by hexokinase, and then it produces pyruvate through glycolysis as well as abundant reduced nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate through the PPP[17]. NADPH serves as a cofactor for glutathione reductase and maintains the active state of antioxidants by converting oxidized glutathione [i.e., glutathione disulfide (GSSG)] to glutathione (GSH)[14]. Ribose-5-phosphate is necessary for nucleic acid synthesis[14]. Pyruvate is metabolized to lactate in the cytosol or converted to acetyl coenzyme A (acetyl-CoA) in the mitochondria to fuel the tricarboxylic acid (TCA) cycle, enhance OXPHOS, and generate ATP[18,19]. Under hypoxic conditions, pyruvate is converted into lactate during glycolysis[20].

Cancer cells adapt to the tumor environment by reprogramming various metabolic pathways to meet their high-energy demands. The upregulation of specific nutrient transporters increases cellular entry of glucose and amino acids[21]. However, the generation of ATP as an energy source in the mitochondria induces oxidative stress caused by increased ROS production, resulting in cell death[11,22,23]. In mitigating oxidative stress, glucose metabolism is rewired to the PPP that operates parallel to glycolysis to maintain redox homeostasis by generating ribose-5-phosphate and NADPH[11]. Cancer cells prefer aerobic glycolysis over OXPHOS to support their proliferation, which is known as the Warburg effect[22,23]. Pyruvate is irreversibly converted to acetyl-CoA by pyruvate dehydrogenase (PDH) in the mitochondria. Pyruvate dehydrogenase kinase (PDK) inhibits PDH activity[24]. Thus, PDK suppresses the metabolic shift from glycolysis to the TCA cycle by downregulating PDH, thereby reducing mitochondrial ROS production and suppressing cell death[24,25]. Furthermore, cancer cells upregulate the PPP, an early diverging branch of glycolysis, thereby promoting NADPH production and exerting antioxidant defenses[26]. Cancer cells not only switch from OXPHOS to lactate-dependent energy-generating pathways, but also upregulate antioxidant-related genes [e.g., NF-E2-related factor 2 (Nrf2)] to overcome a wide range of oxidative stress[27]. Furthermore, NRF2 accelerates cellular redox homeostasis by upregulating the transcriptional regulation of multiple NADPH-generating enzyme genes (e.g., G6PD)[26]. Cancer cells rely not only on glucose, but also on glutamine for their energy demands[25]. Glutaminase is a critical enzyme that converts glutamine to glutamate in the mitochondria[28]. Glutamate contributes to GSH synthesis and maintains redox homeostasis through reduced ROS generation[29]. Apart from glycolysis in cancer cells, glutaminolysis (i.e., a series of biochemical reactions by which glutamine is lysed to glutamate) is another main pillar for energy production, including the conversion of glutamine to α-ketoglutarate (αKG), reaction steps of the citric acid cycle and malate aspartate shuttle, and the conversion of malate to pyruvate and lactate. Glutaminolysis increases OXPHOS and compensates for the energy deficiency in glycolytic cancer cells[25].

In addition, altered metabolism or metabolic rewiring facilitates the adaptation of cancer cells to changing external environments, and this adaptation may render certain nutrients indispensable, a process known as nutrient addiction[15]. Glutamine[30] and cystine addiction have been found in renal cell carcinoma[31], breast cancer[32], and non-small cell lung cancer[15,33]. HGSC and CCC are highly sensitive to cystine-deprived death, exhibiting a cystine addiction phenotype[15,34,35]. In addition, heterogenous tumors are composed of multiple subpopulations associated with different proliferative and malignant potentials[36]. For example, specific tumor types have different metabolic features; primary tumors build biomass such as glucose to sustain their high proliferative demands; metastatic tumors rely on pyruvate, glutamine, and lipid metabolism, and cancer stem cells depend on mitochondrial metabolisms such as OXPHOS and aerobic glycolysis[12,37]. Such a nutrient addiction can be targeted for therapy because many cancer cells have limited energy and nutrient flexibility[25,26,38].

THE ROLE OF FERROPTOSIS IN CANCER

Eukaryotic cells produce energy in the form of ATP and generate ROS as a byproduct. Such cells have evolved an array of antioxidant mechanisms, such as the thiol system, to combat oxidative stress, including nitric oxide, carbon monoxide, and hydrogen (per)sulfide[27]. Cancer cells have also evolved the antioxidant defense system to protect themselves from excess ROS, but oxidative stress exceeding the antioxidant defense mechanism leads to cell death. Cell death is divided into two forms, namely, accidental cell death and regulated cell death[39]. The latter is subdivided into apoptotic and non-apoptotic cell death. The non-apoptotic cell death includes autophagy, ferroptosis, pyroptosis, and necroptosis[40]. Ferroptosis is characterized by the iron-dependent accumulation of excessive ROS and lipid peroxides, leading to cell death[15,35,41-44]. Excess iron in ovarian cancer amplifies free oxidative radicals and drives the Fenton reaction, thereby inducing ferroptosis[7,15]. However, HGSC is characterized by ferroptosis resistance because it can acquire sufficient antioxidant capacity. For example, Nrf2, a representative antioxidant gene, activates the transcription of ferritin heavy chain 1 (a protein involved in iron storage) and heme oxygenase-1 (a protein involved in heme breakdown) to reduce labile iron and regulate iron metabolism and oxidative stress[15,45]. These antioxidants block ferroptosis by limiting cellular oxidative stress and lipid peroxidation.

In general, ferroptosis is regulated by membrane transporter expression, metabolic flexibility, and nutrient dependency. First, solute carrier family 7 member 11 (SLC7A11), commonly known as xCT, is a cystine/glutamate antiporter[19]. The xCT pathway plays an important role in antioxidant defense by transporting extracellular cystine into cells and converting cystine to cysteine for GSH biosynthesis and ROS detoxification[16,46-48]. GSH is synthesized from three constituent amino acids, namely, cysteine, glycine, and glutamic acid, with cysteine being a rate-limiting precursor. Glutathione peroxidase 4 (GPX4) utilizes GSH to convert lipid hydroperoxides into nontoxic lipid alcohols, thereby preventing ferroptosis[19]. HGSCs are characterized by xCT overexpression along with the activation of GSH biosynthesis[48]. Such a metabolic landscape suggests that HGSC depends on cystine uptake to counteract high levels of iron-dependent oxidative stress and maintain redox homeostasis, thereby preventing ferroptosis-induced cell death[16]. Therefore, the inhibition of xCT-mediated cystine transport, cystine depletion, limited cysteine biosynthesis, impaired GSH synthesis, or inactivation of GPX4 can induce ferroptosis[49]. During ferroptosis, the following breakdown products of lipid peroxides are formed, for example, malondialdehyde (MDA), 4-hydroxynonenal, 4-hydroxyhexenal, and 4-oxo-nonenal, and oxidized and modified proteins[50]. High serum levels of MDA have been reported in patients with ovarian cancer compared with healthy women[51], indicating the occurrence of ferroptosis. Such breakdown products may be evaluated as potential surrogate biomarkers for ferroptosis.

Second, ferroptosis is significantly influenced by metabolic flexibility and nutrient dependency. A large amount of NADPH generated via the PPP is consumed to synthesize cysteine and GSH[26]. Thus, cancer cells must evolve mechanisms to cope with NADPH depletion. For example, cancer cells upregulate the expression level of the PPP[26], NRF2[52], and isocitrate dehydrogenase 1 (IDH1)[48] as backup mechanisms for NADPH production. Nrf2 enhances the cellular antioxidant defense through NADPH regeneration[53]. NADPH is also produced during the $NADP^+$-dependent conversion of isocitrate to alpha-ketoglutarate (αKG) by IDH1[48]. These backup mechanisms facilitate the survival and proliferation of cancer cells[26,48,52]. Therefore, drugs that block the uptake of specific nutrients may provide potential therapeutic opportunities to kill cancer cells that rely on the same metabolic pathway[54]. Therapeutic strategies targeting nutrient addiction or metabolic vulnerabilities may induce ferroptosis and inhibit tumor growth. Strategies targeting metabolic vulnerabilities will not only eliminate backup systems to prevent ferroptosis[49], but also receive considerable attention in cancer therapeutics[54].

CURRENT UNDERSTANDING OF METABOLIC PATHWAYS INVOLVED IN FERROPTOSIS

Glucose, glutamine, and fatty acids are major metabolic fuels to meet nutritional demands[16]. Tumor cells receive energy supplies from unique metabolic pathways such as glycolysis, PPP, glutaminolysis, and OXPHOS; upregulate NADPH and GSH production; and downregulate ROS production to control ferroptosis[14]. First, we summarized the mechanism by which glucose regulates ferroptosis in the context of energy stress[18]. Under intact PPP and glycolysis with ample glucose and amino acid supply, NADPH can support the xCT-mediated cystine uptake[14,48]. Free cystine accumulated in cancer cells forms a water-insoluble toxic crystal, often leading to cell damage (i.e., disulfide stress)[48]. Cystine crystals induce ROS production through increased disulfide stress and promote oxidative stress reactions within cancer cells[48]. NADPH can suppress intracellular cystine-dependent disulfide stress by converting cystine into cysteine for GSH synthesis[14,48]. Thus, the PPP-generated NADPH rescues xCT-overexpressing cancer cells (xCT^{high} cancer cells) from ferroptosis, demonstrating that xCT^{high} cancer cells become highly dependent on the glucose-PPP pathway (i.e., NADPH addiction) to inhibit ferroptosis [Figure 1A].

On the contrary, peritumoral angiogenesis cannot keep up with rapidly proliferating lesions, resulting in regions of low blood supply. Such tumor cells must survive under glucose deprivation-induced metabolic stress conditions[14,55]. Glucose starvation decreases carbon flux, which leads to the depletion of PPP and NADPH, inhibition of the conversion of cystine to cysteine, and marked accumulation of cystine, a disulfide molecule[46,56]. xCT-mediated cystine uptake suppresses ferroptosis under conditions with sufficient glucose supply; however, its effect might be limited under glucose starvation, suggesting that glucose starvation may be associated with increased disulfide stress and ferroptosis[48,56] [Figure 1B]. Sufficient glucose supply prevents ferroptosis in cancer cells, whereas glucose deprivation promotes cell death, indicating that cystine addiction determines the survival or death of cancer cells[48]. In addition, limited glucose supply results in cell death in xCT^{high} cancer cell lines, including breast, cervical, kidney, and glioblastoma[48,57]. Further experiments validated that the downregulation of xCT expression suppresses the promoting effect of cancer cell death under nutritional deficiency[58]. However, persistent chronic glucose starvation in surviving ovarian cancer cells results in phenotypic changes through metabolic plasticity, leading to the acquisition of drug resistance and cancer relapse[59].

Second, we summarized the mechanism by which energy stress positively and negatively regulates ferroptosis. Recent research has focused on a potential link between AMP-activated protein kinase (AMPK), a critical sensor of cellular energy status, and ferroptosis[55]. Energy stress caused by glucose starvation activates AMPK[55], and the activated AMPK circumvents metabolic stress by restoring energy balance[60]. AMPK inactivates lipogenic genes, such as acetyl-CoA carboxylase (ACC), and it could inhibit ferroptosis[55]

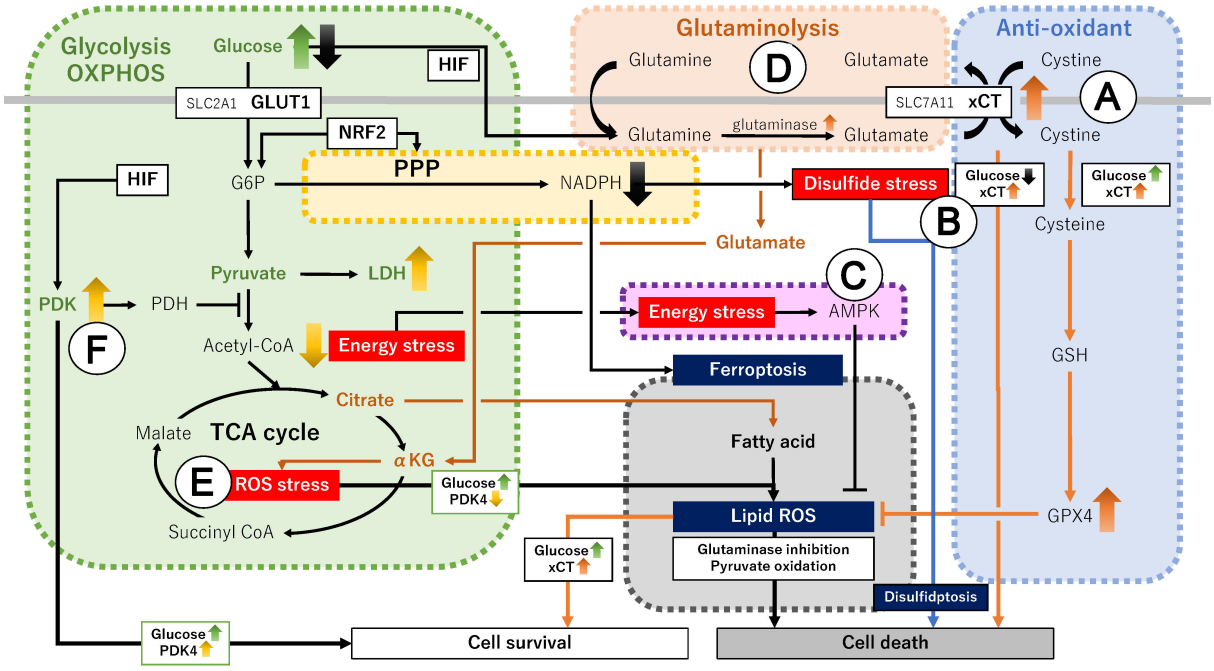

Figure 1. Metabolic pathways involved in ferroptotic cell death. Green, yellow, brown, purple, blue and gray boxes indicate glycolysis and OXPHOS, PPP, glutaminolysis, energy stress, antioxidant, and ferroptosis pathways, respectively. αKG: α-ketoglutarate; AMPK: AMP-activated protein kinase; G6P: glucose 6-phosphate; GLUT1/SLC2A1: glucose transporter 1; GPX4: glutathione peroxidase 4; GSH: glutathione; HIF: hypoxia-inducible factor; LDH: lactate dehydrogenase; NADPH: nicotinamide adenine dinucleotide phosphate; NRF2: NF-E2-related factor 2; OXPHOS: oxidative phosphorylation; PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; PPP: pentose phosphate pathway; ROS: reactive oxygen species; SLC7A11: solute carrier family 7 member 11; TCA: tricarboxylic acid; xCT: cystine/glutamate antiporter SLC7A11.

[Figure 1C]. However, conflicting data have also been reported, that is, AMPK promotes ferroptosis[61]. AMPK triggers the formation of a beclin 1 (BECN1)-SLC7A11 complex via BECN1 phosphorylation[61]. The binding of BECN1 to SLC7A11, a core component of xCT, inhibits the xCT function and promotes ferroptosis[62], indicating that BECN1 is a key ferroptosis inducer[63]. In xCT- and BECN1-overexpressing cancer cells, AMPK phosphorylates BECN1, induces binding to SLC7A11, and promotes ferroptosis by directly blocking xCT activity[64]. Therefore, AMPK either inhibits ferroptosis in response to glucose starvation or promotes ferroptosis by blocking xCT activity. The expression level of ACC or BECN1 in AMPK-activated cancer cells may be critical in determining cell fate. AMPK has also been reported to promote tumor growth by alleviating energy stress or to suppress tumor growth by inhibiting key metabolic pathways such as glucose, glutamine, and fatty acid biosynthesis[65]. These findings suggest that energy stress exerts inhibitory and promoting effects during ferroptosis, but the detailed mechanism remains unclear. Recent studies have reported that AMPK-activating drugs may affect metabolic plasticity[66].

Third, we summarized the mechanism by which glutamine metabolism is involved in ferroptosis, particularly on xCT. Under glucose deprivation, tumor cells rely on an alternative energy-generating pathway such as glutaminolysis[25,67] [Figure 1D]. In meeting the high-energy demand, tumor cells express high levels of glutamine transporters and glutamine synthase, often resulting in glutamine addiction[13,25,29]. Glutamate is converted to αKG, an intermediate in the TCA cycle in glutaminolysis, which activates the TCA cycle, although much remains exported by xCT in exchange for extracellular cystine[16]. Therefore, the loss of cellular glutamate could reduce αKG production and suppress TCA cycle activities, thereby leading to reduced lipid ROS levels and decreased ferroptosis sensitivity[16]. This process may be a characteristic of

xCT^{high} cancer cells. Over time, xCT imports extracellular cystine in exchange for intracellular glutamate release. Glutaminolysis supplies cancer cells with adequate glutamate to maintain extracellular cystine uptake[16,26,46]; however, the accumulated excess intracellular cystine induces disulfide stress, leading to the overproduction of ROS and gradually promoting ferroptosis[16]. Furthermore, sustained cystine uptake leads to extracellular cystine depletion, suppressing glutamate export and promoting intracellular glutamate accumulation. Glutamate may be converted to αKG to maintain TCA cycle function and subsequently enhance the production of lipid ROS to induce ferroptosis[16,68]. In particular, high-OXPHOS ovarian cancer cells were demonstrated to utilize glutamine, reconfirming the importance of glutamine[69]. Therefore, ferroptosis sensitivity may depend on the glucose starvation status, the level of intracellular NADPH, αKG, or glutamine and extracellular cystine, and the expression level of xCT in each cancer cell[16,26,46,58].

In addition, energy stress such as the depletion and overproduction of ATP in cancer cells determines cell fate. The mitochondria are the main sites for ATP generation through OXPHOS, producing ROS as a byproduct of cellular metabolism[13,70]. In cysteine-depleted cancer cells, the TCA cycle activated by αKG induces cellular lipid ROS levels, thereby promoting cell death[71] [Figure 1E]. The moderate suppression of the TCA cycle and OXPHOS reduces mitochondrial ROS production and inhibits cell death[71]. However, extreme ATP depletion (i.e., energy deprivation) induces cell death. That is, energy stress can induce cell death by ATP depletion (i.e., energy deprivation) and ATP overproduction (e.g., excess ROS generation caused by increased αKG). In addition, homologous recombination-deficient cancer cells require ATP for poly(ADP-ribose) polymerase (PARP)-dependent DNA repair mechanisms used in ovarian cancer treatment, demonstrating that ATP causes sensitivity to PARP inhibitors[72].

Fourth, we summarized the mechanism by which metabolic reprogramming of glycolysis and OXPHOS regulates ferroptosis. Hypoxia is the major cause of the rapid growth of cancer cells. Metabolic reprogramming is essential for the adaptation of cancer cells to a hypoxic microenvironment[73]. HIF-1α overexpressed under hypoxic conditions contributes to aggressive phenotypes in tumor cells[13]. There are two types of energy production from glucose: glycolysis and mitochondrial OXPHOS[74]. HIF-1 activates glycolysis-related genes, including pyruvate dehydrogenase kinase 1 (PDK1), increasing the conversion of glucose to pyruvate and subsequently to lactate by inactivating PDH, which is considered critical for metabolic adaptation to hypoxia[74] [Figure 1F]. PDK4 has been reported to block a metabolic switch from glycolysis to predominantly fatty acid synthesis and contribute to ferroptosis resistance in certain cancer cells, such as pancreatic ductal adenocarcinoma (PDAC) cells[18,19]. PDK1 can also mediate a metabolic shift from mitochondrial OXPHOS to glycolysis and increase the proliferation and angiogenesis in ovarian cancer xenografts[75]. Therefore, the Warburg effect has a profound impact on ferroptosis pathways through PDK-dependent metabolic switches or metabolic adaptations in cancer.

Fifth, we focused on the role of the tumor suppressor p53 in the pathophysiological process of ferroptosis in ovarian cancer. p53 induces essential biological processes such as cell cycle arrest, senescence, DNA repair, apoptosis, autophagy, and the reprogramming of cellular metabolism[38]. p53 has been reported to regulate various metabolic pathways and promote metabolic reprogramming to induce drug resistance and metastasis[38]. In particular, p53 inhibits SLC7A11 expression and reduces glutathione synthesis, making cancer cells susceptible to oxidative damage and sensitive to iron by increasing lipid peroxide levels[32,76,77]. In a previous study, Zhang *et al.* reported that p53 facilitates ferroptosis in ovarian cancer cells treated with iron oxides[76].

Sixth, a tight interplay between ferroptosis and translation has attracted increasing attention in the field of cancer research. Translation initiation machinery, such as initiation factors and ribosomal proteins,

modulates gene regulation during nutrient deprivation and metabolic stress[78]. The upregulation of mammalian target of rapamycin (mTOR), a master regulator of translation initiation, results in the increased expression of cancer-promoting genes such as eIF4E, a limiting factor for translation initiation in most cancers, including ovarian cancer[79,80]. The activation of mTOR leads to the phosphorylation of eukaryotic translation initiation factor 4E-binding protein (4E-BP), increased recruitment of eukaryotic translation initiation factor 4E (eIF4E), and initiation of protein translation[81]. eIF4E plays a key role in many physiological processes such as protein synthesis, cell growth, proliferation, angiogenesis, and carcinogenesis. eIF4E was found to inhibit aldehyde dehydrogenase (ALDH) activity and increase the ferroptosis sensitivity of ovarian cancer cells by accumulating lethal lipid peroxidation[82,83]. The ALDH enzyme family detoxifies ROS-mediated lipid peroxidation-generated aldehydes such as MDA. In addition, the stimulation of GPX4 protein synthesis was enhanced through the mTOR/eIF4E axis[84,85]. Therefore, the dysregulation of translational machinery alters susceptibility to ferroptosis in ovarian cancer cells. Collectively, the mTOR signaling pathway is involved in many crucial biological processes, including ferroptosis, as it is frequently activated in a wide range of tumors, including ovarian cancer.

Finally, cancer cells require not only glucose and glutamine metabolism in the tumor microenvironment, but also iron metabolism to maintain cell survival[9]. Iron plays an important role in ferroptosis, and ovarian cancer is characterized by high intracellular iron content[6]. Ovarian cancer cells upregulate transferrin receptor (the iron importer) and downregulate ferroportin (the iron efflux pump), indicating increased iron uptake[7]. That is, cancer cells increase iron supply and decrease iron loss based on a unique phenotype of iron metabolism[9]. Therefore, a literature search was performed to determine whether the metabolic pathway characteristic of cancer cells affects iron transport and iron metabolism associated with ferroptosis. Only one paper showed that glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in glycolysis, is involved in iron metabolism as a transferrin-binding protein, independent of its canonical role in glycolysis[86]. Iron plays an important physiological role, including oxygen transport and energy metabolism, but little is known about whether energy metabolism affects iron homeostasis in ovarian cancer.

THERAPEUTIC STRATEGY BASED ON FERROPTOSIS

Despite the advances in the treatment of ovarian cancer, many patients experience intrinsic and acquired resistance to anticancer drugs, which have poor outcomes. Several recent in vitro and animal studies have shown that ferroptosis inducers, chemotherapy, and immunotherapy can synergistically affect ovarian cancer[35,41]. In this study, two potential treatment options targeting ferroptosis were investigated: The first treatment option directly induces ferroptosis and its downstream pathways, and the other targets metabolic vulnerabilities associated with ferroptosis, such as glycolysis, glutaminolysis, and energy stress dependence. The key molecules associated with the former include xCT, GSH, GPX4, intracellular labile iron, and ferroptosis-inhibitory pathways. Cancer has evolved several regulatory mechanisms of ferroptosis, which neutralize ROS and prevent lipid peroxidation, including the xCT-GSH-GPX4 axis[15,42,44], ferroptosis-suppressor-protein 1 (FSP1)-coenzyme Q10 (CoQ10)-NADPH axis[87], and Hippo pathway[88]. Several studies have also reported on xCT molecular-targeted therapy for in vivo application. Considering that highly sensitive xCT inhibitors and cystine deprivation can induce ferroptosis, several ferroptosis-inducing agents have been developed[15,35,42]. Some recent reviews have provided evidence for the association between ferroptosis inhibition and ovarian cancer progression, discussing the potential therapeutic application of ferroptosis-inducing agents[15,35,42-44]. On the contrary, the latter is a therapeutic strategy that induces ferroptosis by targeting rewired energy metabolism and its potential metabolic compensation[31,41]. The suppression of specific metabolism may represent an attractive therapeutic strategy for the treatment of ovarian cancer[41]. Therapeutic strategies targeting the ferroptosis pathway and metabolic vulnerabilities associated with ferroptosis have been reported in breast cancer and other types of cancer[13,25]. In this review,

therapeutic strategies were divided into "a therapeutic strategy focusing on the ferroptosis pathway" and "a therapeutic strategy focusing on metabolic vulnerability and nutrient addiction." However, mechanisms such as "disulfide stress" do not apply to either strategy.

Therapeutic strategy focusing on the ferroptosis pathway

SLC7A11, a molecule that protects cancer cells from ferroptosis-induced cell death, is overexpressed in different types of cancers, including ovarian cancer, lung cancer, triple-negative breast cancer, PDAC, renal cell carcinoma, liver cancer, and glioma, and it is associated with aggressive phenotypes and poor prognosis[16,46,89]. Ferroptosis resistance involves the sustained overexpression of xCT and activation of its downstream signaling. The upregulation of xCT induces oxidative stress resistance and protects against lipid peroxidation[90]. xCT inhibitors have drawn increasing attention because of their antitumor effect on ovarian cancer using preclinical animal models[91]. Class 1 ferroptosis inducers, including erastin, imidazole ketone erastin, sulfasalazine, sorafenib, and HG106, directly inhibit xCT activity[92,93] and induce ferroptosis by preventing cystine uptake and depleting cysteine or GSH, thereby inducing lipid peroxidation and cell death[15,49]. Several studies have highlighted the importance of elastin in single or combination therapeutic strategies for ovarian cancer. For example, C-Myc amplified in ovarian cancer cells inhibits ferroptosis by inducing NCOA4-mediated ferritinophagy[94], but erastin selectively kills iron-addicted ovarian cancer cells by inducing ferroptosis and promoting NCOA4-mediated ferritinophagy and mitochondrial dysfunction[95]. However, ovarian cancer cells with low intracellular iron pools are resistant to erastin treatment[95], indicating that iron levels can determine cell sensitivity to ferroptosis. Ovarian cancer cells that are less susceptible to ferroptosis may be platinum-resistant[96] and may be clinically recommended for co-treatment with ferroptosis-inducing agents. Erastin synergizes with cisplatin to inhibit ovarian cancer growth through ferroptosis[97]. Erastin sensitizes ovarian cancer cells to wee1 inhibitor AZD1775 and synergistically inhibits their growth[98]. Wee1 is a G2 checkpoint kinase. PARP inhibitors were reported to promote SLC7A11-mediated ferroptosis[99]. However, erastin can facilitate ovarian cancer cell invasion in vivo by inducing IL-8 production and then M2 macrophage polarization[100]. Despite the antitumor effect of erastin, evidence has also shown that iron concentration and macrophage polarization in the tumor microenvironment promote the resistance of ovarian cancer cells to erastin-induced ferroptosis. Although xCT inhibition as a regulator of ferroptosis is a potential strategy for cancer therapy, the potential targets of ferroptosis in the treatment of ovarian cancer in vivo and their mechanisms remain poorly understood. Furthermore, Ras-selective lethal 3 (RSL3) and the 5,6-dihydro-2H-pyrano[3,2-g]indolizine (DPI) class of luminogen (DPI, also known as ML162) are known as class 2 ferroptosis inducers that directly inhibit GPX4 enzymatic activity[91,93]. RSL3 induces ferroptosis by inhibiting the GPX4 activity in drug-resistant ovarian cancer cells[101]. Apart from class 1 and class 2 ferroptosis inducers, targeting molecules associated with ferroptosis in ovarian cancer is an emerging field of therapeutics.

In addition, ferroptosis is characterized not only by the loss of GPX4 activity and subsequent accumulation of labile iron and excessive ROS production, but also by the peroxidation of polyunsaturated fatty acids (PUFAs)[102,103]. The inhibition of GPX4 causes the aberrant accumulation of PUFA hydroperoxides. In general, acyl-CoA synthetase long-chain family member 4 (ACSL4), lysophosphatidylcholine acyltransferase 3 (LPCAT3), and lipoxygenases (e.g., 15-LO) promote the peroxidation of phospholipids containing PUFA during ferroptosis[104]. Zhao *et al.* summarized several inhibitors targeting different enzymes in the lipid metabolism network (e.g., ACC, fatty acid synthase, sterol regulatory element-binding protein 1, and stearoyl-CoA desaturase) and discussed targeting lipid metabolism in ovarian cancer[104]. Therefore, the role of lipid peroxidation in ferroptosis in several human cancers, including ovarian cancer, has been emphasized[44,104]. Targeting lipid metabolism may be an important and potential strategy in cancer therapy; thus, combining drugs that modulate ferroptosis with conventional cancer therapies has received significant interest[104]. Table 1 shows the agents that affect the ferroptosis pathway by modulating the accumulation of iron, ROS, and lipid peroxides.

Table 1. Agents that affect the ferroptosis pathway by modulating the accumulation of iron, ROS, and lipid peroxides

Agents			
The biological function or action of the agents	**Official symbol/Official full name**	**Function**	**Ref.**
The accumulation of iron			
NRF2 inhibition	NRF2/Nuclear factor erythroid 2-related factor 2	NRF2 decreases the labile iron pool and increases ferroptosis resistance through controlling HERC2 [HECT and RLD domain containing E3 ubiquitin protein ligase 2; E3 ubiquitin ligase for NCOA4 and FBXL5 (F-box and leucine-rich repeat protein 5)] and VAMP8 (vesicle-associated membrane protein 8; mediating autophagosome-lysosome fusion).	[105]
HMOX1 inhibition	HMOX1/Heme oxygenase 1	HMOX1 is the enzyme responsible for degradation of heme and generates antioxidant and anti-inflammatory byproducts. Upregulation of HMOX1 inhibits ferroptosis and promotes ovarian cancer cell growth.	[106]
Ferritinophagy/Autophagic degradation of ferritin		Ferroptosis vulnerability is induced through autophagic degradation of ferritin (i.e., ferritinophagy) of ferritin heavy chain 1 (FTH1) in cisplatin-resistant ovarian cancer.	[107]
Iron nitroprusside		Iron nitroprusside is thought to be an effective treatment for ovarian cancer because it induces lipid peroxidation via the Fenton reaction and subsequently promotes ferroptosis.	[45]
The accumulation of ROS			
SLC7A11 degradation	HRD1/E3 ubiquitin ligase 3-hydroxy-3-methylglutaryl reductase degradation	Promoting ubiquitination-dependent SLC7A11 degradation	[108]
FZD7 inhibition	FZD7/Wnt receptor Frizzled 7	FZD7 reduces ferroptosis sensitivity through the β-catenin-Tp63-GPX4 pathway in platinum-resistant ovarian cancer cells.	[109]
FSP1 inhibition	FSP1/Ferroptosis suppressor 1	FSP1 is a GSH-independent suppressor of ferroptosis that acts as an NADH-dependent CoQ10 oxidoreductase, contributing to ferroptosis resistance via reducing CoQ10.	[15]
The Hippo effectors	YAP/Yes-associated protein 1; TAZ/Transcriptional coactivator with PDZ-binding motif	The Hippo effectors YAP and TAZ induce ferroptosis in ovarian cancer cells through overexpressing Angiopoietin-Like 4 (ANGPTL4) and NADPH Oxidase 2 (NOX2). The Hippo proteins control cell fate.	[110]
NRF2 modulator	NCTD/Norcantharidin	NCTD, a normethyl compound of cantharidin, facilitates ferroptosis by inhibiting the NRF2/HO-1/GPX4/xCT axis.	[111]
The accumulation of lipid peroxides			
SCD1 inhibition	SCD1/Stearoyl-CoA desaturase 1	SCD1 catalyzes the formation of monounsaturated fatty acids (MUFAs), specifically oleate and palmitoleate. Inhibition of SCD1 causes iron-mediated lipid peroxidation and mitochondrial dysfunction by downregulating GPX4 and then induces ovarian cancer cell death. SCD1 inhibitor co-treatment may enhance the antitumor efficacy of ferroptosis inducers in ovarian cancer.	[112-114]
ACSL1 inhibition	ASCL1/Acyl-CoA synthetase long-chain family member 1	ACSL1 reduces the level of lipid peroxidation and enhances ferroptosis resistance in ovarian cancer through increasing the stability of FSP1.	[115]
Others			
Ferroptosis-related gene	PRNP/Prion protein	Overexpression of ferroptosis-related gene prion protein (PRNP) inhibits ovarian cancer proliferation and invasion.	[116]
Ropivacaine		A local anesthetic ropivacaine induces ferroptosis in ovarian cancer cells by inactivating the PI3K/Akt pathway.	[117]

GPX4: Glutathione peroxidase 4; SLC7A11: solute carrier family 7 member 11.

Therapeutic strategy focusing on metabolic vulnerabilities and nutrient addiction

In response to dynamically changing nutrient availability in the tumor microenvironment, cancer cells facilitate cellular adaptations to reprogram metabolic pathways. Therefore, metabolic vulnerabilities are attacked using the synthetic lethality approach in ovarian cancer cells overexpressing xCT, rather than directly inhibiting xCT or GPX4. Cancer cell-specific metabolic pathways or nutrient addiction, such as

glycolysis, glutaminolysis, or other energy stress dependence, can also be utilized as therapeutic targets. However, it is difficult to assess in real time which metabolic pathways cancer cells are currently most dependent on. Despite recent progress in the development of xCT inhibitors, effective treatment interventions focusing on metabolic vulnerabilities remain unmet.

First, xCT inhibitors such as erastin have emerged as an effective treatment option to facilitate ferroptosis in a high-glucose tumor environment[19]. Ferroptosis has been reported to be dependent on glucose uptake by GLUT 1 (also known as SLC2A1)[18]. The maintenance of glycolysis in xCT^{high} cancer cells is essential for constituting a potential treatment strategy to induce ferroptosis [Figure 2A]. By contrast, glucose deprivation induced by GLUT inhibitors selectively blocks xCT inhibitor-induced ferroptosis, thereby suppressing cancer cell death[18]. That is, xCT inhibitors may exhibit anticancer properties against cancer cells with elevated GLUT1 expression in a high-glucose tumor environment, whereas glucose deprivation blocks ferroptosis-induced cell death[18,19] [Figure 2B]. xCT inhibitors show contrasting effects in the presence or absence of glucose in the tumor microenvironment.

Second, the PPP-generated NADPH plays a central role in the cellular metabolic network and redox homeostasis[14]. NADPH can support the biosynthesis of GSH, thioredoxin, and CoQ10 to boost the antioxidant defense in cancer cells and protect cancer cells from ferroptosis and cell death[14] [Figure 3A]. Therefore, targeting the PPP in tumor cells may provide a therapeutic strategy based on ferroptosis. Glucose deprivation reduces the PPP-mediated NADPH generation, making it impossible to reduce insoluble cystine imported via xCT to a more soluble cysteine, thereby inducing disulfide stress (i.e., cystine-dependent toxicity) and leading to rapid cell death[48] [Figure 3B]. This finding suggests that xCT-overexpressing cancer cells are sensitive to glucose starvation-induced cell death. Furthermore, cysteine depletion in CCC abolishes glycolysis and OXPHOS and inhibits cancer cell proliferation[118], indicating that cysteine depletion caused by the decreased conversion of cystine to cysteine plays a critical role in cancer therapy. Therefore, not only the GLUT inhibitor but also the glucose-6-phosphate dehydrogenase inhibitor or 6-amino-nicotinamide, a nicotinamide analog, may be used as an inhibitor of the PPP. Collectively, these studies suggest that xCT is beneficial for cancer cells by suppressing ferroptosis, while glucose deprivation and NADPH depletion caused by a reduction in carbon from glucose entering the PPP can promote cancer cell death[26,48].

Third, PDK4 inhibits the TCA cycle and reduces the production of PUFAs to enhance ferroptosis resistance[18] [Figure 4A]. Dichloroacetate, a PDK inhibitor, induces the metabolic switch from glycolysis to OXPHOS to generate ROS accumulation, thereby facilitating ferroptosis[119] [Figure 4B]. Erastin can mediate ferroptosis through mitochondrial voltage-gated anion channels (VDAC) and xCT, which impairs VDAC function, thereby resulting in mitochondrial dysfunction, ROS production, and cell death[120]. Thus, xCT inhibitors enhance mitochondrial respiration and increase ROS production to promote ferroptosis, which may be a potential therapeutic strategy in cancer cells exhibiting metabolic reprogramming from glycolysis to OXPHOS. Furthermore, co-treatment with PDK inhibitors and erastin may synergistically enhance ferroptosis in cancer cells overexpressing GLUTs and xCT[18].

Fourth, glutaminase mediates the conversion of glutamine to glutamate, leading to the entry of glutamine into the TCA cycle. Cancer cells upregulate and consume glutamine to produce metabolic fuel for cancer cell proliferation and redox status regulation [Figure 5A]. Cancer cells may be more sensitive to glutaminase inhibition under high glutamine import compared with that under low glutamine import[25]. xCT-overexpressing cancer cells are sensitive to glutaminase inhibition because the inhibition of glutamine metabolism decreases GSH production and increases ROS production[13,121] [Figure 5B]. xCT not only

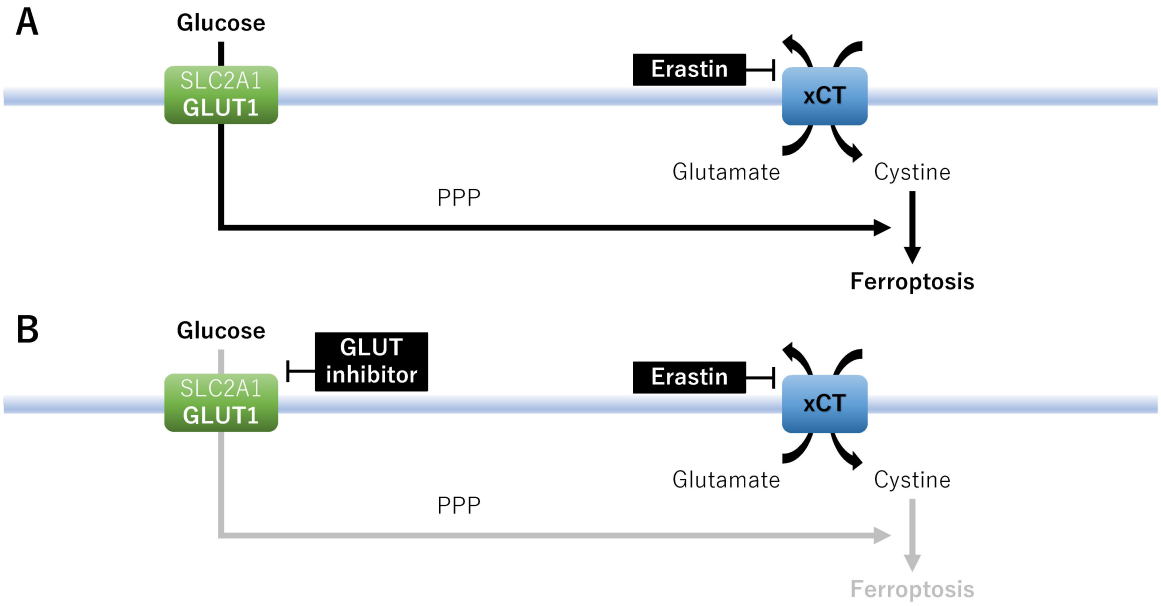

Figure 2. A therapeutic strategy focused on metabolic vulnerabilities and nutrient addiction: glucose metabolism. Effects of erastin on ferroptosis in the absence (A) or presence (B) of GLUT inhibitors. GLUT1/SLC2A1: Glucose transporter 1; PPP: pentose phosphate pathway; xCT: cystine/glutamate antiporter SLC7A11.

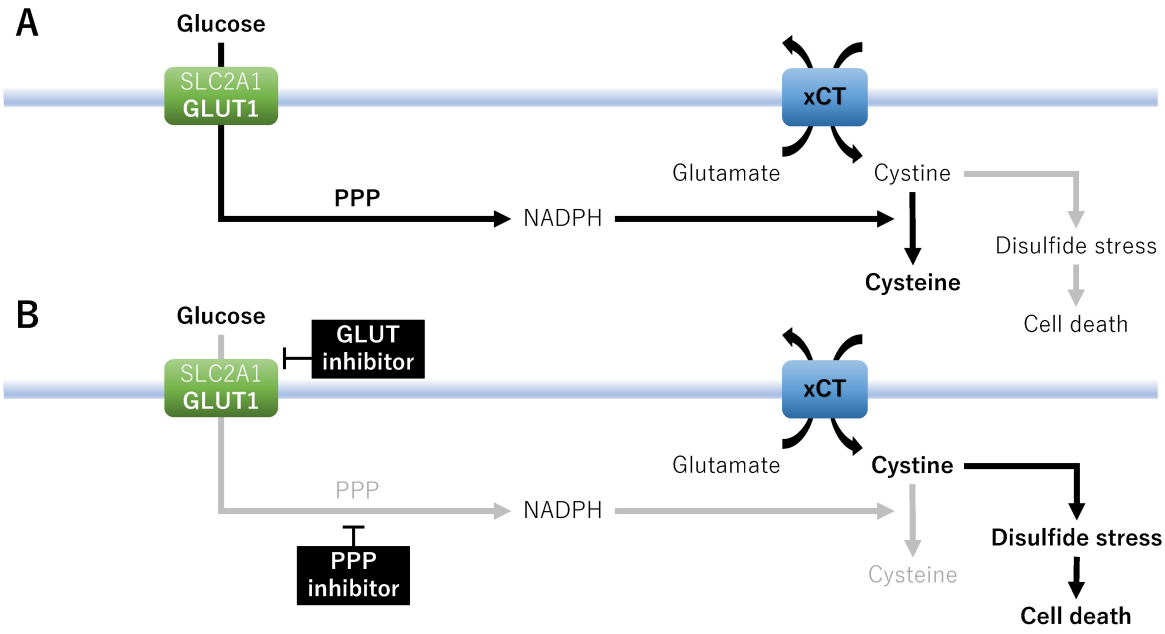

Figure 3. A therapeutic strategy focused on metabolic vulnerabilities and nutrient addiction: the PPP metabolism. Effects of xCT on cell death in the absence (A) or presence (B) of GLUT inhibitors or PPP inhibitors. GLUT1/SLC2A1: Glucose transporter 1; NADPH: nicotinamide adenine dinucleotide phosphate; PPP: pentose phosphate pathway; xCT: cystine/glutamate antiporter SLC7A11.

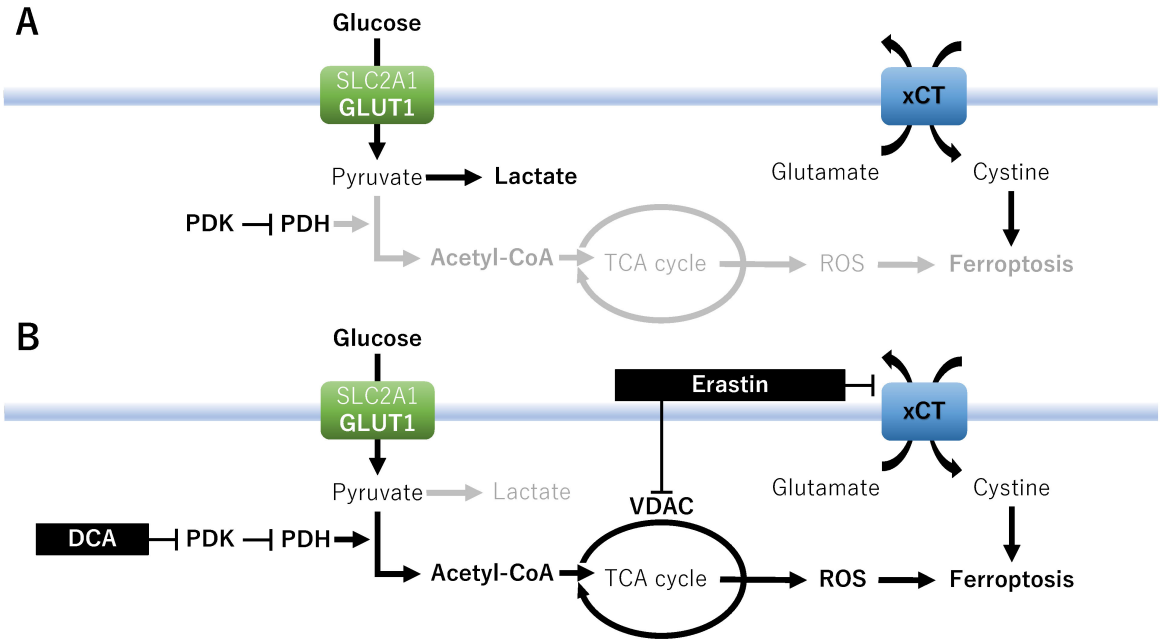

Figure 4. A therapeutic strategy focused on metabolic vulnerabilities and nutrient addiction: PDK-dependent metabolism. Effects of xCT on ferroptosis in the absence (A) or presence (B) of erastin or PDK inhibitor. DCA: Dichloroacetate; GLUT1/SLC2A1: glucose transporter 1; PDK: pyruvate dehydrogenase kinase; PDH: pyruvate dehydrogenase; ROS: reactive oxygen species; TCA: tricarboxylic acid; VDAC: voltage-gated anion channels; xCT: cystine/glutamate antiporter SLC7A11.

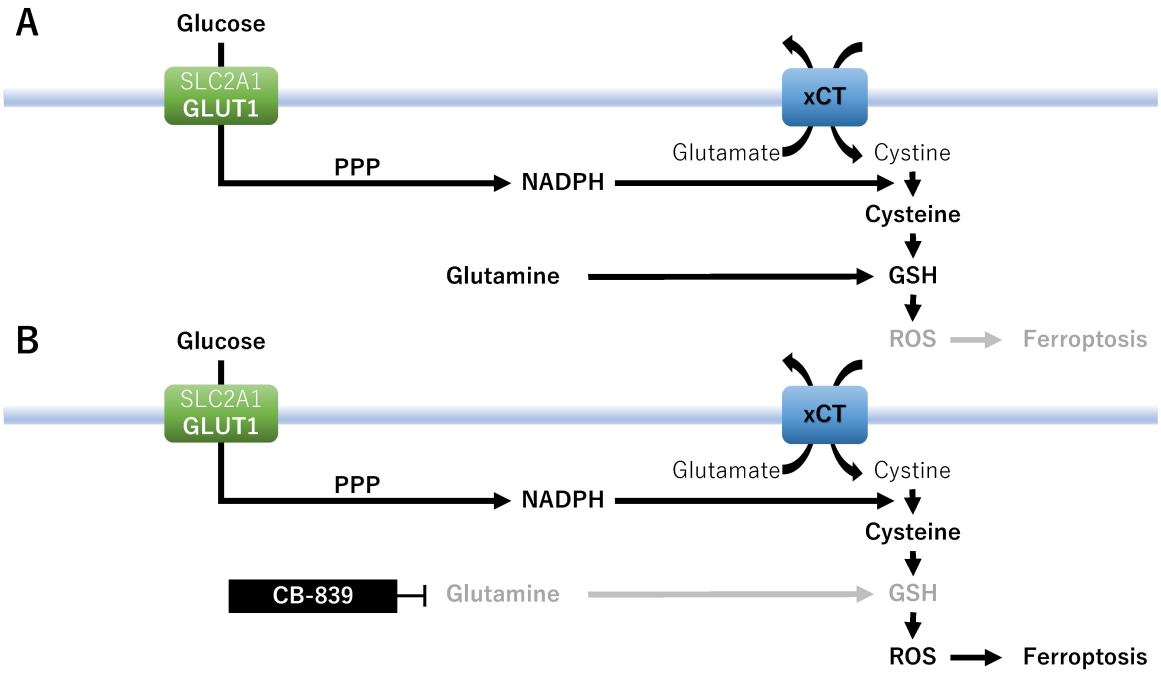

Figure 5. A therapeutic strategy focused on metabolic vulnerabilities and nutrient addiction: glutamine metabolism. Effects of xCT on ferroptosis in the absence (A) or presence (B) of glutaminase inhibitor. GLUT1/SLC2A1: Glucose transporter 1; GSH: glutathione; NADPH: nicotinamide adenine dinucleotide phosphate; PPP: pentose phosphate pathway; ROS: reactive oxygen species; xCT: cystine/glutamate antiporter SLC7A11.

catalyzes glutamate release, but also regulates glutamine uptake[16]. xCT-overexpressing cancer cells lead to glutamine dependency, which presents potential metabolic vulnerabilities as therapeutic targets. A recent review has discussed new breast cancer treatment strategies based on glutaminase modification, leading to cellular ferroptosis[25]. CB-839 (telaglenastat), a glutaminase inhibitor, blocks tumor glutamine consumption[122]. A phase 1 clinical study will assess the safety, tolerability, and preliminary evidence of the antitumor activity of CB-839 in combination with the mTOR inhibitor sapanisertib (MLN0128)[122].

Finally, some drugs or natural compounds have been reported to have antitumor properties by targeting metabolic vulnerabilities such as alterations in glycolysis, OXPHOS, and glutamine metabolism[13]. For example, Yung *et al.* reported that dual-targeted therapy utilizing AMPK-activating drugs and vascular endothelial growth factor/programmed cell death 1 blockade may be a new treatment option for ovarian cancer focusing on metabolic vulnerability[66]. A natural-occurring enzyme, glucose oxidase, converts glucose into non-metabolizable gluconic acid and H_2O_2, amplifying the ferroptotic damage. A bioactive protein, MAP30, isolated from bitter melon seeds, triggered apoptosis and ferroptosis in various ovarian cancer cells[123]. Furthermore, research on compounds derived from natural products may lead to the development of new treatment options that promote ferroptosis.

DISCUSSION

In this review, the regulatory mechanism of ferroptosis through multiple metabolic pathways was summarized, and therapeutic strategies targeting ferroptosis in ovarian cancer were discussed. In particular, ovarian cancer is strongly influenced by an iron- and ROS-dependent mode of cell death, namely, ferroptosis, from the early stages of carcinogenesis. Changes in iron metabolism, lipid peroxidation responses, and a wide range of energy metabolism play important roles in regulating ferroptosis sensitivity of ovarian cancer cells[124]. Two therapeutic strategies targeting xCT were highlighted in this review, that is, directly inhibiting xCT activity and targeting glucose/glutamine dependency as a therapeutic vulnerability in xCT-overexpressing cancer cells. New perspectives for improving cancer therapy based on ferroptosis were also discussed. xCT inhibitors (e.g., erastin) can induce cell death when carbon from glucose, glutamine, or fatty acids is abundant[16]. Conversely, nutrient starvation, such as glucose and glutamine, causes cancer cell death because of dysfunction of the xCT-associated antioxidant system. For example, glucose deprivation induces rapid NADPH depletion, promoting ferroptosis-mediated oxidative stress and cell death[16]. Glucose-depleted cancer cells may display glutamine addiction, resulting in the survival of such cells. Elevated glutamine metabolism makes cancer cells more susceptible to glutamine deficiency caused by glutaminase inhibitors, which rapidly induces cell death[122]. Drugs that block intrinsic and acquired nutrient addiction may promote the susceptibility of cancer cells to ferroptosis[16,57]. Therefore, therapeutic approaches that use nutrient addiction to target metabolic vulnerabilities may be effective in overcoming ferroptosis resistance.

Moreover, preclinical studies revealed that ferroptosis inducers contribute to the enhanced efficacy of immunotherapy[125] and chemotherapy[126]. For example, PARP inhibitors sensitize ovarian cancer cells to ferroptosis by synergistically activating ATM/ATR and causing DNA damage[126]. The enhancement of cancer efficacy by eliminating drug resistance may be achieved through synergistic combinations of ferroptosis and existing therapeutic approaches[15]. Thus, cancer therapy that induces ferroptosis could be an innovative therapeutic strategy in ovarian cancer[35,41]. However, xCT has been demonstrated to play contradictory roles in ferroptosis regulation in a tumor-promoting or suppressive manner, depending on changes in energy and nutrient metabolic pathways[48,57]. xCT functions as a double-edged sword to modulate various types of cancer survival and death by regulating the redox balance, nutrient dependency, energy stress, and ferroptosis process[16]. Furthermore, the role of metabolic flexibility and vulnerability in

regulating xCT-mediated ferroptosis was summarized, and the current understanding of ferroptosis-induced therapy in ovarian cancer was discussed. Ferroptosis inducers should be used on the basis of the metabolic characteristics of cancer cells.

CONCLUSION AND FUTURE PERSPECTIVES

Cells have evolved mechanisms to maintain redox homeostasis through metabolic reprogramming whenever they encounter a large burden of oxidative stress resulting from changes in the microenvironment[127]. Cancer cells are forced to alter their energy and nutrient metabolic pathways to adapt to ever-changing environmental changes[127]. Cancer cells acquire autonomous capabilities for tumor promotion by upregulating xCT activity, inducing antioxidant defenses, and suppressing ferroptosis[16,46]. The key molecules in signaling pathways associated with glycolysis (e.g., PDK4), OXPHOS (e.g., IDH1 and αKG), glutaminolysis (e.g., glutaminase), PPP (e.g., G6PD and NADPH), and P53 pathway are critically involved in regulating ferroptosis in ovarian cancer[12]. xCT-overexpressing cancer cells depend on glucose, glutamine, and fatty acids for an energy source to acquire proliferative and survival advantages[16,17,19,68]. All main metabolic pathways, including glycolysis, TCA cycle, glutaminolysis, OXPHOS, and PPP, are individually altered in ovarian cancer cells[13].

Several reliable molecular biomarkers can predict cell death associated with ferroptosis. However, the biopsy of tumor tissue has its own limitations. Liquid biopsy provides a minimally invasive diagnostic modality to assess the molecular characterization of the tumor and to allow a personalized approach to patients with effective treatment in real time[128]. Liquid biopsy of the blood and peritoneal fluid in patients with recurrent ovarian cancer may be used as a drug screening platform to select potential drugs. The gene expression profile can be verified by reverse transcription-polymerase chain reaction assay using customized pre-selected genes. This panel should include candidate genes potentially associated with ferroptosis, for example, SLC7A11, GLUT1, PDK, PDH, glutaminase, GPX, BECN1, ACC, and AMPK. xCT inhibitors can also have therapeutic benefits for ovarian cancer cells growing in a high-glucose tumor environment (e.g., ovarian cancer cells overexpressing SLC7A11, GLUT1, PDK, glutaminase, and GPX). By contrast, drugs that target metabolic pathways (e.g., GLUT1 inhibitors, PPP inhibitors, PDK inhibitors, and glutaminase inhibitors) may provide promising efficacy in ovarian cancer cells harboring nutrient addiction. Therefore, drugs can be selected on the basis of the expression pattern of ferroptosis-inducing genes (e.g., SLC7A11, GSH, and GPX) or genes associated with metabolic pathways that affect ferroptosis (e.g., GLUT, PDK4, glutaminase, VDAC, and G6PD). Such gene expression patterns may serve as biomarkers for selecting patients with cancer for personalized treatment. Further studies based on the regulation of xCT expression, ROS stress and redox homeostasis, and energy stress caused by specific nutrient addiction or deficiency will increase the clinical importance of ferroptosis modulation as an effective therapeutic strategy for ovarian cancer.

DECLARATIONS

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Validation: Yoshimoto C, Shigetomi H, Imanaka S
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Cancer Drug Resistance

Review

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Targeting metabolic vulnerabilities to overcome resistance to therapy in acute myeloid leukemia

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Abstract

Malignant hematopoietic cells gain metabolic plasticity, reorganize anabolic mechanisms to improve anabolic output and prevent oxidative damage, and bypass cell cycle checkpoints, eventually outcompeting normal hematopoietic cells. Current therapeutic strategies of acute myeloid leukemia (AML) are based on prognostic stratification that includes mutation profile as the closest surrogate to disease biology. Clinical efficacy of targeted therapies, e.g., agents targeting mutant FMS-like tyrosine kinase 3 (FLT3) and isocitrate dehydrogenase 1 or 2, are mostly limited to the presence of relevant mutations. Recent studies have not only demonstrated that specific mutations in AML create metabolic vulnerabilities but also highlighted the efficacy of targeting metabolic vulnerabilities in combination with inhibitors of these mutations. Therefore, delineating the functional relationships between genetic stratification, metabolic dependencies, and response to specific inhibitors of these vulnerabilities is crucial for identifying more effective therapeutic regimens, understanding resistance mechanisms, and identifying early response markers, ultimately improving the likelihood of cure. In addition, metabolic changes occurring in the tumor microenvironment have also been reported as therapeutic targets. The metabolic profiles of leukemia stem cells (LSCs) differ, and relapsed/refractory LSCs switch to alternative metabolic pathways, fueling oxidative phosphorylation (OXPHOS), rendering them therapeutically resistant. In this review, we discuss the role of cancer metabolic pathways that contribute to the metabolic plasticity of AML and confer resistance to standard therapy; we also highlight the latest promising developments in the field in translating these important findings to the clinic and discuss the tumor microenvironment that supports metabolic plasticity and interplay with AML cells.

Keywords: OXPHOS, DHODH, leukemia stem cells, mesenchymal stromal cells, IDH

INTRODUCTION

Acute myeloid leukemia (AML) is a cancer derived from the myeloid lineage of blood cells. It is characterized by overproduction of leukemic blasts and maturation arrest. With approximately 120,000 cases a year globally, AML is the most common type of acute leukemia in adults[1]. Although conventional chemotherapy is known to eliminate bulk tumor cells, its utility is limited in AML, as older patients lack tolerance to chemotherapy and the drug may lack efficacy at eliminating leukemia stem cells (LSCs). Residual clones that survive chemotherapy lead to disease relapse[2]. Moreover, AML exhibits extensive genetic heterogeneity, as it often harbors a complex mixture of heterogenous subclones that possess genetic aberrations in diverse driver genes, with distinct clonal evolutionary patterns existing in single individuals. Cytogenetic abnormalities and acquired somatic mutations are used as surrogate markers for risk assessment and some mutations can be specifically targeted. However, the utility of therapies that target mutations viz. FMS-like tyrosine kinase 3 (FLT3) and isocitrate dehydrogenase enzyme 2 (IDH2) are limited to the context of the particular mutation.

Cancer cells are known to reprogram their metabolism to support their survival and proliferation; this was recently recognized as a hallmark of cancer[3]. AML cells exhibit unique metabolic dependencies compared with those of their normal counterparts, the noncancerous blasts. Hematopoietic stem cells (HSCs) use mitochondrial oxidative phosphorylation (OXPHOS) during differentiation to meet their higher energy requirements; however, they use anaerobic glycolysis for their routine energy needs and generally avoid aerobic mitochondrial OXPHOS[4]. Of note, mitochondrial OXPHOS generates reactive oxygen species (ROS), which are deleterious for HSCs. In fact, ROS functions as a signaling mediator in the crosstalk between metabolism and stem cell fate decisions by inhibiting the repopulating capacity of HSCs[5]. ROS-high-HSCs are characterized by low self-renewal capacity and high myeloid differentiation capacity compared to ROS-low HSCs.

Upon cell division, HSCs have fate choices when they undergo cell division; they either undergo self-renewal, wherein they produce new HSCs, or differentiate, wherein they produce cells that mature into committed cells. On the basis of the status of the daughter cells, HSC division can occur in one of the following ways: (1) asymmetric division, which maintains the HSC pool, in which one daughter cell remains as stem cell while the other differentiates; (2) symmetric commitment, in which both daughter cells differentiate (stem cell exhaustion); or (3) symmetric division, in which two daughter stem cells are produced, which helps in HSC expansion. Interestingly, mitochondria distribution during stem cell division appears to be crucial to determining the fate of HSCs. Of note, mitochondrial division is asymmetrical during stem cell division and daughter cells that receive fewer old mitochondria maintain stem cell traits[6]. Notably, through a ROS-mediated physiological process, changes in mitochondrial dynamics regulate stem cell fate decisions, triggering a dual program to suppress self-renewal and promote differentiation[6,7]. Evasion from mitochondrial OXPHOS and reliance on glycolysis help prevent HSC pool exhaustion. In contrast, LSCs rely on mitochondrial OXPHOS to generate high-energy compounds while maintaining ROS levels at non-toxic levels by employing a wide repertoire mechanism for ROS mitigation[8]. Therefore, it may be possible to target leukemic cells differently based on their metabolic needs for therapeutic purposes.

In this review, we discuss the role of cancer metabolic pathways in AML development that contribute to the metabolic plasticity of the disease and confer resistance to standard therapy. We also highlight the latest developments in the field including clinical trials for translating metabolic inhibitors to clinic [Figure 1] and discuss the role of tumor microenvironment and extracellular vesicles in supporting metabolic plasticity and their interplay with AML cells.

Drug		Clinical trial			Condition/Disease	Trial status	
Name	Description	Identifier	Phase	Purpose		Recruitment	Results
Targeting oxidative phosphorylation (OXPHOS)							
IACS-010759	ETC complex I inhibitor	NCT02882321		DEE	Recurrent AML		
ONC201	ClpP	NCT02392572		Safety, best dose and efficacy	Relapsed or Refractory Acute Leukemias and MDS		
CPI-613	lipoate analog taregts TCA	NCT05854966		Response rate of CPI-613 and metformin	Relapsed/Refractory AML		
Metformin	biguanide	NCT01849276		MTD for Metformin + Cytarabine	Relapsed/Refractory AML		
Targeting amino acid metabolism							
CB-839[a]	Glutaminase Inhibitor	NCT02071927		Safety, PK, PD, biomarkers, and efficacy	Newly diagnosed or relapsed AML		
Erwinase	Asparaginase	NCT02283190		Safety and reduction of serum glutamine	AML		
GRASPA[b]	L-asparaginase	NCT01810705		Efficacy and tolerability	AML unfit for intensive chemotherapy		
PEG-BCT-100[c]	Arginase (PEGylated)	NCT02899286		Reduction of serum glutamine	Relapsed or refractory AML		
ADI-PEG 20	Arginase (PEG based)	NCT01910012		Reduction of serum glutamine	AML		
Targeting pyrimidine biosynthesis							
BAY2402234	DHODH inhibitor	NCT03404726		Safety, tolerability, PK, and efficacy	AML, MDS, or CMML		
Brequinar	DHODH inhibitor	NCT03760666		Safety, tolerability, and efficacy of brequinar	Relapsed or Refractory AML		
ASLAN003	DHODH inhibitor	NCT03451084		Efficacy	Relapsed/refractory AML patients		
JNJ-74856665	DHODH inhibitor	NCT04609826		DEE	AML/MDS		
PTC299	DHODH inhibitor	NCT03761069		Safety and PK, and efficacy	Relapsed/Refractory Acute Leukemias		
Targeting mutant IDH1 and IDH2							
Enasidenib (AG-221)	Inhibitor of IDH2	NCT02577406		Safety and efficacy	IDH2 mutant refractory or relapsed AML		
Ivosidenib (AG-120)	Mutant IDH1 inhibitor	NCT03173248		Safety and efficacy	Untreated AML with an IDH1 Mutation		
IDH305	Mutant IDH1 inhibitor	NCT02381886		Maximum tolerated dose / recommended dose	AML with IDH2 mutation		
BAY-1436032	Mutant IDH1 inhibitor	NCT03127735		Maximum tolerated dose / recommended dose	Advanced AML with IDH1-R132X mutation		
AG-881	Mutant IDH1/2 inhibitor	NCT02492737		Safety, PK, PD, and efficacy	Advanced hematologic cancers with mutant IDH1/2		
LY3410738	Oral, covalent IDH inhibitor	NCT04603001		DEE; PD, PK, MTD, safety and efficacy	Advanced hematologic cancers with mutant IDH1/2		
FT-2102[c]	Inhibitor of mutated IDH1	NCT02719574		Safety, efficacy, PK and PD	AML, MDS with mutant IDH1		

Clinical trail: Phase I, Phase II, Phase III

Recruitment: Recruiting, Not recruiting, Completed, Terminated

Results: Not posted, Posted

[a]CB-839 in combination with Azacytidine;
[b]L-asparaginase encapsulated in erythrocytes I combination with cytarabine in AML;
[c]FT-2102 alone or in combination with azacytidine or cytarabine
DEE, Dose escalation and expansion;
PK, Pharmacokinetics,
PD, Pharmacodynamics,
DEE, Dose escalation and expansion,
MTD, Maximum tolerated dose

Figure 1. List of clinical trials for metabolic inhibitors in AML. AML: Acute myeloid leukemia; ClpP: caseinolytic protease proteolytic subunit; CMML: chronic myelomonocytic leukemia; DHODH: dihydroorotate dehydrogenase; ETC: electron transport chain; IDH: isocitrate dehydrogenase enzyme; MDS: myelodysplastic syndromes; OXPHOS: oxidative phosphorylation; TCA: tricarboxylic acid cycle.

TARGETING MITOCHONDRIAL OXPHOS

AML cells are more sensitive to mitochondria-targeted drugs, likely because despite having increased respiratory activity, their mitochondria have lower coupling efficiency and spare reserve capacity due to proton leak[9]. In different cancers, including AML, depletion of mtDNA results in OXPHOS deficiency. OXPHOS-deficient cancer cells fail to form tumors unless mtDNA is restored from stromal cells via the horizontal transfer of whole mitochondria[10-13], suggesting that functional OXPHOS is essential for cancer development, although it is not clear which component of OXPHOS contributes the most to tumor formation. For instance, aspartate supplementation enables cells to proliferate under pharmacological inhibition of electron transport chain (ETC), suggesting that although adenosine triphosphate (ATP) production is the primary function of OXPHOS, proliferating cancer cells obtain oxidizing power and aspartate for pyrimidine biosynthesis through aerobic respiration[14,15], thus, in addition to ATP or ROS levels, there may be other mechanisms underlying the antiproliferative effects of ETC inhibition. Several compounds block complex I (i.e., NADH: ubiquinone oxidoreductase), with high affinity viz. IACS-010759[16], IM156[17], BAY87-2243[18], and ME-344[19].

The OXPHOS inhibitor IACS-010759 is currently being evaluated in phase 1 clinical trials in relapsed/refractory AML [Figure 1][16]. BAY87-2243, an inhibitor of hypoxia-inducible factor-1 (HIF-1) that inhibits mitochondrial complex I activity, causes grade III nausea/vomiting [NCT01297530], while ME-344 showed

no clinical efficacy in patients with solid cancers[18,20].

Oxidative phosphorylation integrity is maintained by a protease, caseinolytic protease proteolytic subunit (ClpP) in AML cells[21]. The altered function of this protease contributes to misfolding or degradation of respiratory chain subunits and their accumulation, causing respiratory chain dysfunction. The imipridones ONC201 and ONC212 inhibited the growth and viability of leukemia cells by hyperactivating mitochondrial ClpP and thus selectively proteolyzing certain mitochondrial matrix proteins[21]. A significant reduction in the leukemic burden and improved survival was observed in mice that were administered ONC201 orally[21]. In a clinical trial, a single oral dose of ONC201 resulted in a decrease in circulating blasts and a subsequent increase in platelet counts in patients with relapsed/refractory AML[21].

Mubritinib (TAK-165) is a direct and ubiquinone-dependent ETC complex I inhibitor which induces ROS accumulation. Mubritinib also decreases ATP/ADP and NAD/NADH concentration ratios and inhibits the activities of PDH, tricarboxylic acid cycle (TCA), and OXPHOS, which results in the death of AML cells treated with mubritinib[22]. In vivo, mubritinib delayed AML development without affecting normal hematopoiesis in mice[22]. An isoquinoline alkaloid, berberine, targets mitochondrial ETC complex I, leading to reduced mitochondrial activity and enhanced antileukemic effects in vitro and in vivo when combined with IDH1 mutant inhibitor AG-120[23].

A rationally designed lipoic acid analog, CPI-613, is currently being investigated in clinical trials for patients with pancreatic cancer and acute myeloid leukemia[24]. Mechanistically, CPI-613 inhibits the TCA cycle by displacing lipoic acid, a cofactor for the TCA cycle enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. This displacement, in turn, prevents the enzymes' activity and halts the TCA cycle[25].

Metformin is being evaluated in combination with chemotherapy in different cancers; it sensitizes AML cells to Ara-C by reducing the mitochondrial transfer from bone marrow stromal cells (BMSCs) and OXPHOS in the recipient cells[26]. Metformin was found to synergistically sensitize AML cells to Ara-C by inhibiting the mammalian target of rapamycin (mTOR)C1/P70S6K pathway[27]. By promoting autophagy and apoptosis mediated by mTOR, metformin sensitizes FLT3-ITD-positive acute myeloid leukemia to sorafenib[28]. Additionally, metformin and phenformin are also potentiators of the cytotoxicity of Bcl2 antagonist - ABT-737 against leukemia[29]. By inhibiting protein production, metformin downregulates antiapoptotic Mcl-1 and Bcl-xl expression, resulting in a synergistic anti-tumor effect with ABT-199 in AML[30]. It also exhibited anticancer activity with the MCL1 inhibitor S63845 via redox remodeling[31]. IM156 is another oral biguanide OXPHOS inhibitor of mitochondrial ETC Protein Complex 1, which activates AMPK and is well tolerated at doses active in preclinical models with modest clinical efficacy [NCT03272256] in solid cancer patients[32]. Though the biguanides showed promising efficacy in preclinical studies, they are still under development and warrant further investigation in clinical trials for AML to establish their clinical utility.

Role of mitochondrial OXPHOS in chemoresistance

Cytarabine remains the backbone of AML chemotherapy. Interestingly, pre-existing and persisting cytarabine-resistant cells do not necessarily reside in a quiescent or LSC compartment but rather have higher OXPHOS levels, with polarized mitochondria and an increased mitochondrial mass[33]. The ability of cytarabine to induce mitochondrial ROS is a major determinant of cytarabine sensitivity in primary AML cells. Resistance to cytarabine is often mediated by SIRT3, a NAD+-dependent protein deacetylase that deacetylates mitochondrial anti-oxidant enzymes, including IDH2 and superoxide dismutase 2 (SOD2)[34]. SIRT3-mediated deacetylation increases the enzymatic activity of its antioxidant targets, thereby restricting

cytarabine-induced mitochondrial ROS production and resulting in reduced apoptosis and increased chemoresistance[34]. In addition, SIRT3 drives OXPHOS and increases the levels of both dihydronicotinamide adenine dinucleotide phosphate (NADPH) and reduced glutathione (GSH) while decreasing the concentrations of their respective oxidized forms. SIRT3 activation conferred resistance to chemotherapy *in-vivo* and its inhibition showed a synergistic effect with cytarabine[34]. SIRT3 SUMOylation was higher in Ara-C sensitive primary cells, but lower in resistant primary cells[35]. De-SUMOylation of SIRT3 is mediated by SUMO-specific peptidase 1 (SENP1), which enhances its deacetylase activity by inhibiting its protein degradation. When SIRT3 is de-SUMOylated, the expression of HES1, a negative regulator of FAO, is decreased and FAO is upregulated; either FAO inhibitors or overexpression of HES1 can attenuate this effect[35]. The use of Momordin-Ic a, SENP1 inhibitor or HES1 overexpression in vitro and in vivo showed synergy with cytarabine to eradicate AML cells[35]. Although these studies demonstrate a role for SIRT3 in chemoresistance in AML, the number of patients included in these studies is limited, and therefore validation is required in larger cohorts.

Notably, following cytarabine therapy, residual cells showed increased FAO, increased CD36 expression, (a fatty acid translocase) and higher expression of an *OXPHOS* gene signature that is predictive of clinical response to treatment in PDX models and AML patients[33]. Inhibition of mitochondrial protein biosynthesis, electron transfer, or FAO resulted in reduced OXPHOS and strikingly increased efficacy of cytarabine in AML[33]. Another mechanism of cytarabine resistance is mediated by upregulation of CD39, an ectonucleotidase that is localized on the cell surface and hydrolyses extracellular ATP and ADP to produce adenosine. Increased activity of CD39 promotes mitochondrial activity and biogenesis by activating the cAMP-mediated adaptive mitochondrial stress response, which promotes resistance to cytarabine. The activation of cAMP-PKA signaling driven by CD39 through P2RY13 purinergic receptor causes metabolic reprogramming and promotes the survival of chemo-resistant AML cells[36]. Thus, CD39-P2RY13-cAMP-OxPHOS axis plays a key role in cytarabine resistance; inhibiting CD39 ecto-ATPase activity enhanced cytarabine's cytotoxicity in AML by blocking the mitochondrial reprogramming caused by the drug[36].

TARGETING IDH1/2M

The isocitrate dehydrogenases enzymes, IDH1 and IDH2, catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) in the cell cytoplasm and mitochondria, respectively, and reduce NADP+; this, in turn, contributes to the generation of NADPH. α-KG is essential for the optimal functioning of multiple metabolic and epigenetic processes, while NADPH functions as a reducing agent and plays a crucial role in maintaining the cellular redox state. IDH1/2 mutations have been found in 1 of every 5 AML patients with a higher preponderance among patients with normal karyotypes[37,38]. IDH1/2 mutations detected in AML are predominantly heterozygous point mutations that not only render the protein incapable of carrying out decarboxylation of isocitrate, but confer it the ability to produce 2-hydroxyglutarate (2-HG), an oncometabolite, by NADPH-dependent reduction of α-KG[39]. 2-HG inhibits several α-KG-dependent dioxygenases, such as demethylases and the TET family of 5-methlycytosine hydroxylases[40]. TET regulates the gene expression of its target genes through DNA demethylation and chromatin modification, such as H2B O-linked N-acetylglycosylation (O-GlcNAcylation) or H3K4 trimethylation[41]. The loss of α-KG in conjunction with the suppression of α-KG-dependent dioxygenases mediated by 2-HG promotes leukemogenesis[42,43]. In addition, loss of NADPH due to IDH mutations perturbs the regulation of redox balance in the cell[44].

In recent years, ivosidenib (AG120), an IDH1 inhibitor, has been used in the treatment of relapsed and refractory AML[45] while many other inhibitors including Olutasidenib (FT-2102), IDH305, Vorasidenib (AG881), BAY1436032, LY3410738 and DS-1001 are currently being evaluated in Phase I/II clinical trials in

patients with IDH1/2 mutant AML. The IDH2 inhibitor Enasidenib (AG-221) and Ivosidenib are approved for relapsed/refractory AML in the presence of relevant mutations and ivosidenib was recently approved, in combination with hypomethylating agents, as first-line therapy for IDH1 mutant AML [Figure 1]. The IDH1 mutant AML cells carrying IDH1R132H, IDH1R132C, IDH1R132G, IDH1R132L and IDH1R132S mutations undergo myeloid differentiation when treated with BAY1436032, a novel pan mutant IDH1 inhibitor which specifically inhibits R-2HG production and colony growth in these cells. Aside from that, the compound affects DNA methylation and attenuates hypermethylation of histones. In two independent xenograft mouse models derived from AML patients with IDH1 mutation, treatment with BAY1436032 increased leukemic blast clearance, myeloid differentiation, and stem cell depletion while prolonging survival[46]. Combining azacitidine and BAY1436032 increased survival and depleted LSCs by inhibiting MAPK/ERK and RB/E2F signaling[47]. In a study conducted by Shih *et al.*, AG-221 treatment reduced aberrant hypermethylation in several genes involved in hematopoietic differentiation, suggesting an epigenetically driven differentiation effect. As a single agent, AG221 or 5-azacitidine in vivo stimulated the production of mature myeloid cells from mutant leukemic progenitor blasts. Importantly, in $Idh2^{R140}QFlt3^{ITD}$ mutant AML combination therapy with FLT3 inhibitor AC220 (quizartinib), significantly decreased mutant cell burden and led to the recovery of normal hematopoiesis from non-mutant stem/progenitor cells[48].

The emergence of second-site IDH2 mutations in trans, at glutamine 316 (Q316E) and isoleucine 319 (I319M), which are located at the interface of the enasidenib binding site in the IDH2 dimer, were found to confer therapeutic resistance to enasidenib. Although the expression of these mutant proteins alone is not sufficient for the synthesis of 2HG, the presence of R140Q mutation in trans produces 2HG, which is resistant to inhibition by enasidenib[49]. Mutations in the IDH dimer-interface in cis were found to confer resistance to ivosidenib in AML[49]. In a high-content shRNA screen of isogenic leukemia cells expressing wild-type and mutant IDH1, Chan *et al.* (2015) found that survival of IDH1/2 mutant cells was highly dependent upon BCL-2 and BCL-W expression[50]. Venetoclax-based therapy was effective in 6 out of 7 patients with IDH1 mutations who had previously been treated with ivosidenib; however, a lower response rate was reported for patients with FLT3-ITD mutations, indicating that venetoclax is an effective salvage therapy in patients who previously received IDH1/2 inhibitors[51]. IDH305 is a selective oral IDH1 inhibitor that specifically targets R132* IDH1 mutation. There is a phase I clinical trial currently being conducted on this drug for the treatment of advanced malignancies, including relapsed/refractory AML and myelodysplastic syndromes (MDS) [NCT02381886][52].

Vulnerabilities in IDH mutated AML

Mutations in IDH1/2 have been reported to confer sensitivity to several chemotherapeutic agents in most cancer types, including colorectal cancer[53], glioma[54,55], cholangiocarcinoma[53,56], and AML[50,57]. Conversely, inhibition of mutant IDH1/2 may counteract the cytotoxic effect of chemotherapeutic drugs[58]. In IDH1/2 mutants, D2HG mediates the downregulation of ATM, a DNA damage response gene. Therefore, IDH1/2 mutant AML cells are sensitive to DNA damage-causing agents, such as daunorubicin, and the PARP inhibitors olaparib and talazoparib, while IDH1/2 mutant inhibitors had a protective effect against these treatments[58]. Intriguingly, IDH1/2 mutation sensitized primary AML cells to ABT199 by inhibiting cytochrome c oxidase (i.e., Complex IV of ETC) by 2HG[50]. The cytochrome c oxidase complex contains two heme moieties (a and a3) and two copper atoms (CuA and CuB)[59]. A binding of (R)-2-HG at or near the binuclear center of heme a3 and CuB inhibits cytochrome c oxidase activity, thereby blocking oxygen reduction at that site[50]. Suppression of cytochrome c oxidase activity results in oxygen deprivation, which triggers activation of BAX/BAK and leads to outer membrane permeabilization and apoptosis. When BAX/BAK are bound to BCL-2, permeabilization is prevented, but ABT-199 disrupts this binding so that IDH1/2 mutant cells die, while wild-type IDH1/2 cells are relatively unaffected[33]. IDH1/2 mutant AML exhibited a

superior response (36%) to BCL-2 inhibition compared to IDH1/2 wildtype (9%)[60]. Patients with IDH-mutant AML show robust responses to venetoclax + azacytidine[61]. This is a great example of a driver mutation that creates a unique metabolic vulnerability[62].

Primary resistance to IDH inhibitors is primarily associated with leukemia stemness, while acquired resistance is often conferred by mutations in the genes belonging to the RUNX1/CEBPA or RAS-receptor tyrosine kinase (TK) pathways or genes such as BCOR, and TET2[63]. These mutations often affect transcription factors that are involved in hematopoietic and myeloid differentiation, particularly RUNX1 and CEBPA. An association was found between co-mutation(s) of RUNX1 at baseline and a lower CR rate with IDH inhibitor[63]. RUNX1 mutations were also the most frequently acquired mutations in relapsed disease. In addition, co-occurrence of CEBPA mutation was associated with a lack of response to IDH inhibitor, and the mutation was also reported to be acquired at relapse[63]. Because of their roles as differentiation factors, RUNX1 and CEBPA mutations may interfere with differentiation signals induced by IDH inhibitors, resulting in clinical resistance[63]. Almost 30% of relapse cases were found to have acquired mutations in either NRAS or KRAS during relapse[63]. Interestingly, Tateishi *et al.* found that IDH1 mutation may cause addiction to NAD+ in solid cancers[64]. The mutant form of IDH1 lowered NAD+ levels through downregulation of the enzyme nicotinate phosphoribosyltransferase (NAPRT1) of the NAD+ salvage pathway and thus sensitized cells to NAD+ depletion when combined with inhibition of nicotinamide phosphoribosyltransferase (NAMPT). Depletion of NAD+ activated AMPK, triggering pro-cell death autophagy, resulting in cell death[64].

TARGETING AMINO ACID METABOLISM

Role of glutamine and glutaminolysis: as cellular building blocks and in ROS mitigation homeostasis

Besides serving as substrates for protein biosynthesis, amino acids participate in the biosynthesis of purine and pyrimidine nucleotides and provide precursors for energy generation. For instance, acetyl-CoA can be generated by the ketogenic amino acids leucine and lysine. Similarly, the glycogenic amino acids alanine and glycine generate pyruvate and TCA cycle intermediates. Glutamine addiction has been reported in some primary AML patient samples and cell lines[65-67], and can drive the TCA cycle by providing glutamate; through glutaminolysis, glutamate is then converted to α-ketoglutarate. In addition, it can serve as a substrate in the synthesis of GSH, a ROS mitigator[66,68] [Figure 2]. Gregory *et al.* demonstrated that inhibition of glutaminase, an enzyme responsible for converting glutamine to glutamate, could perturb GSH balance and adversely affect the redox state in AML[69]. AML development in NSG mice was dramatically inhibited by knocking down the glutaminase gene GLS1, leading to the failure of conversion of glutamine to glutamate (glutaminolysis); this, in turn, induced apoptosis, and exhibited a synergistic effect in sensitizing leukemic cells to venetoclax[66].

Drivers of glutaminolysis

The cellular influx and efflux of glutamine in exchange for leucine influences the intracellular levels of glutamine, which is critical for mTORC1 activity, and translation and control of autophagy to coordinate cell survival and proliferation[70]. Solute-carrier family 1 member 5 (SLC1A5) is responsible for glutamine transport into the cells. Glutamine transport is essentially linked to leucine uptake. The bidirectional transporter, SLC7A5/3A2 is responsible for the uptake of leucine in exchange of glutamine[71]. Knockdown of SLC1A5 in a murine model of AML resulted in increased apoptosis and reduced tumor formation[72].

Translation of cellular mRNAs is tightly regulated by mTOR. The mTOR pathway regulates the phosphorylation of 4E-BP1 (at serine 65), which is required for the initiation of translation[73]. Asparaginases are routinely used in the treatment of acute lymphoblastic leukemia ; they catalyze the conversion of

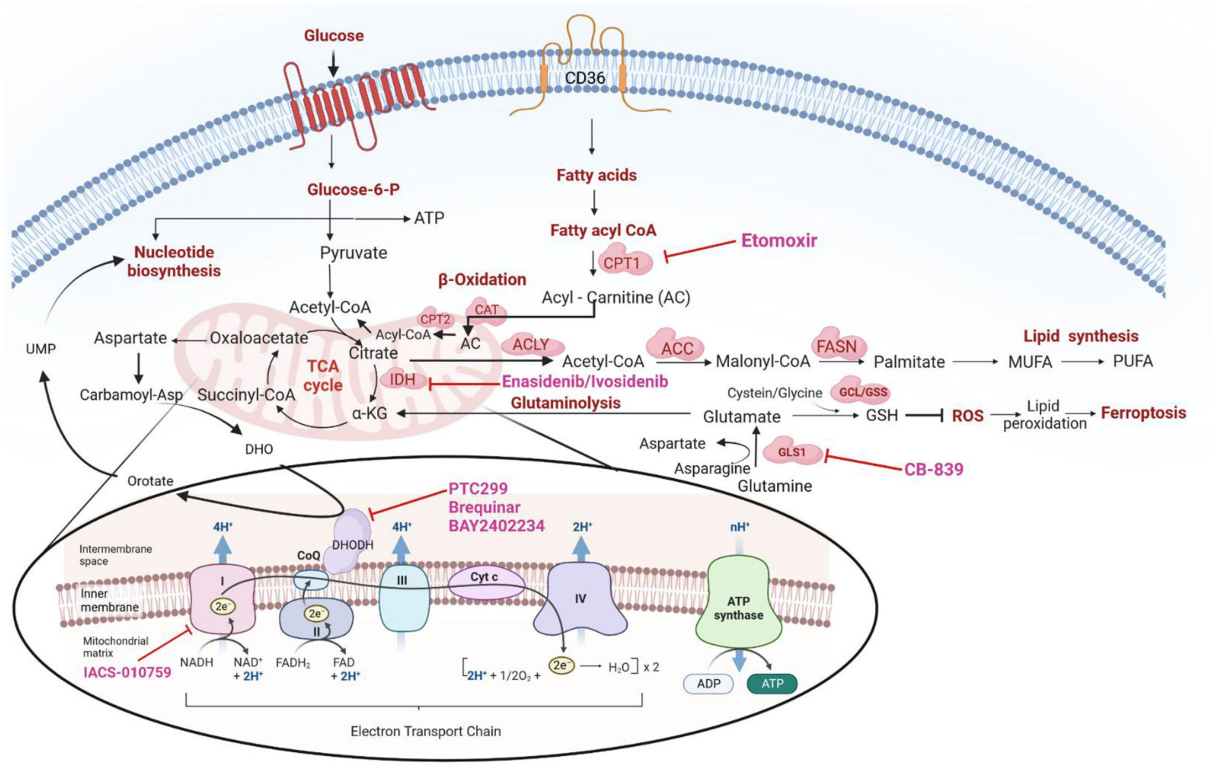

Figure 2. Overview of cellular metabolic pathways and their crosstalk -including: (1) glycolysis; (2) TCA cycle; (3) OXPHOS; (4) glutaminolysis; (5) nucleic acid biosynthesis; (6) lipid synthesis; (7) fatty acid import and oxidation; (8) ROS mitigation by GSH; (9) ferroptosis. ACC: Ac-CoA carboxylase; ACLY: ATP citrate lyase; ADP: adenosine diphosphate; ATP: denosine triphosphate; α-KG: α-ketoglutarate; CAT: carnitine acetyltransferase; CPT1: carnitine palmitoyltransferase 1; CPT2: carnitine palmitoyltransferase 2; DHO: dihydroorotate; DHODH: dihydroorotate dehydrogenase; FAD: flavin adenine dinucleotide; FASN: FA synthase; GSH: glutathione; IDH: isocitrate dehydrogenase enzyme; GCL: glutamate-cysteine ligase; GSS: glutathione synthetase; GLS1: glutaminase 1; NAD: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide (NAD) + hydrogen (H); MUFA: monounsaturated fatty acid; OXPHOS: oxidative phosphorylation; PUFA: polyunsaturated fatty acid; ROS: reactive oxygen species; TCA: tricarboxylic acid cycle; UMP: uridine monophosphate.

asparagine and glutamine to aspartate and glutamate, respectively, and help to reduce the concentrations of asparagine and glutamine in the blood[74]. Glutamine depletion induced by L-asparaginases inhibits 4EBP1 phosphorylation at residue S65 and decreases protein synthesis in AML cell lines[72]. When 4EBP1 is dephosphorylated, it binds to eIF4E, resulting in inactivation of translational initiation complexes and a reduction in cap-dependent protein synthesis. In Complex karyotype AML, co-treatment with venetoclax and pegcrisantaspase, an asparaginase, significantly diminished cellular protein synthesis including that of MCL-1, an important antiapoptotic protein in AML, by promoting the interaction between eIF4E and 4EBP1 on the cap-binding complex and interfering with active cap-mRNA translation downstream of mTOR signaling[75].

Glutaminolysis is a therapeutically targetable vulnerability in FLT3-ITD and other tyrosine kinase (TK) activating leukemias. A synthetic lethality screen using CRISPR/Cas9, metabolomics analyses, and gene expression analysis reveals that FLT3-ITD cells develop glutamine metabolism dependence following FLT3 TK inhibition. FLT3 TK inhibition hinders glucose uptake and use, suppressing the enhanced central carbon metabolism that is found in FLT3-ITD cells and reversing the glycolytic phenotype; as a result, FLT3-ITD cells develop a metabolic dependency on glutamine metabolism. Despite a decrease in glucose uptake, TCA cycle activity and respiratory function are less affected by FLT3 TK inhibition; instead, they are

supported by the continuous uptake of glutamine. FLT3 TK inhibition with concomitant suppression of glutamine metabolism using either GLS chemical inhibition or gene silencing, increased cell death in FLT3-ITD cells[76]. Glutaminolysis is also upregulated by FLT3-ITD through Foxo3a inhibition. Foxo3a activates the expression of MAX-interacting protein 1 (MXI-1), a protein which can block MAX from binding to Myc. This disruption of the interaction between Myc and MAX reduces the transactivation capacity of Myc[77]. In AML and other cancer cells, FLT3-ITD inhibits Foxo3a[77,78], leading to Myc activation and glutaminolysis stimulation, which, in turn, generates anaplerotic substrates that fuel the TCA cycle, increasing citrate synthesis and its transport to cytoplasm. Increased citrate synthesis provides acetyl-CoA, which can feed into the biosynthesis of lipids and participate in the post-translational modification of proteins[44].

Agents targeting glutaminolysis

Glutaminolysis was recently identified as a therapeutic target for hematological and solid malignancies, and potent inhibitors are now being evaluated in clinical trials [NCT02071862 and NCT02071927]. Inhibition of glutaminolysis with CB-839 blocks glutamine utilization, resulting in decreased levels of glutamate, aspartate and several TCA cycle intermediates. As described in a previous study[5], inhibition of glutaminolysis with BPTES in mutant IDH1/2 AML may be beneficial, as it arrested cell proliferation, decreased 2-HG levels and enhanced differentiation[67]. Interestingly, in AML harboring FLT3-ITD mutations, glutaminolysis inhibition alone confers only minor antiproliferative effects. Glutaminolysis inhibition is more effective when combined with FLT3-ITD tyrosine kinase inhibition[76], which perturbs glycolysis and glucose utilization, with less effect on glutamine metabolism. Therefore, even though glycolysis and glucose utilization are impaired following FLT3 inhibition, glutamine can drive the TCA cycle and participate in GSH synthesis[76]. Notably, in AML cells treated with quizartinib, glutamine starvation resulted in the depletion of GSH and elevation of intracellular ROS. In addition to glutamine starvation, pharmacological inhibition using CB839 or glutaminolysis gene silencing enhanced the efficacy of FLT3-ITD tyrosine kinase inhibition by reducing the availability of intracellular glutamine (due to TCA cycle inhibition), and GSH generation, thus impacting redox metabolism[76].

LSCs exhibit increased uptake and catabolism of amino acids. Venetoclax + azacytidine is highly effective in AML patients because of its action at inhibiting amino acid metabolism in LSCs[79]. Jones *et al.* demonstrated that depletion of amino acids, especially cysteine, was strongly associated with response to venetoclax + azacytidine regimen compared to ROS induction. LSCs in patients with de novo AML rely on amino acid metabolism to drive OXPHOS. In LSCs, the depletion of cysteine causes a reduction in GSH levels, which, in turn, leads to decreased glutathionylation of succinate dehydrogenase A (SDHA), a component of ETC complex II. This decreased glutathionylation of SDHA results in OXPHOS inhibition and ATP depletion, ultimately leading to LSC death[80]. BCL-2 inhibition is believed to promote the generation of mitochondrial permeability transition pores, resulting in leakage of mitochondrial membrane and reduced amino acid metabolism[81]. The combination of venetoclax and azacytidine has shown good efficacy in older AML patients, who are generally considered unfit for traditional chemotherapy due to high toxicity and poor outcomes with chemotherapeutic agents. In fact, in a Phase III study, this regimen resulted in higher complete remission compared to the placebo control (36.7% *vs.* 17.9%; $P < 0.001$), the composite complete remission was reported in 66.4% AML patients receiving ven + aza *vs.* 28.3% in placebo control; $P < 0.001$[61]. The study reported that almost half of all patients receiving azacitidine plus venetoclax had a first response (complete remission or complete remission with incomplete hematologic recovery) before starting cycle 2, and their remissions lasted an average of 17.5 months. Of note, the remission rate after venetoclax + azacytidine in patients with *de novo* AML is independent of the genetic background, suggesting that venetoclax targets a unique metabolic program of LSCs[61].

Targeting arginine

Primary AML cells are auxotrophic for glutamine, cysteine, and arginine[80,82]. As they lack argininosuccinate synthetase-1 (ASS1), a key enzyme required for the generation of arginine, these cells rely on arginine import[83]. The depletion of extracellular arginine via the use of pegylated arginine deiminase, which converts plasma arginine to citrulline, induced responses in AML through the activation of caspases[83]. Depleting extracellular arginine shows synergy with cytarabine and enhances its cytotoxicity[82]. Arginine and cysteine metabolism are also controlled by the stroma in CLL, which may promote the resistance and growth of leukemic cells[84]; this can be targeted by arginine depletion.

Targeting branched-chain amino acids

The survival of HSCs and LSCs is influenced by branched-chain amino acids (BCAAs), such as leucine, isoleucine, and valine. For instance, valine is essential for survival and maintenance of HSCs[85,86]. BCAAs promote cancer growth in *de novo* AML[87]. LSCs exhibit elevated levels of branched-chain amino acid transaminase 1 (BCAT1)[88], a cytoplasmic aminotransferase that generates glutamate by transferring an amino group from a BCAA to α-KG[89], which can in turn fuel OXPHOS.

TARGETING PYRIMIDINE BIOSYNTHESIS

Proliferating cells require an abundance of nucleotides. They heavily depend on *de novo* nucleotide biosynthesis to meet their nucleotide requirements. As expected, the expression of enzymes involved in nucleotide biosynthesis is elevated in cancer. Uridine monophosphate (UMP) is generated in *de novo* nucleotide biosynthesis from glutamine. Similarly, dihydroorotate dehydrogenase (DHODH) is a mitochondrial enzyme that converts dihydroorotate (DHO) into orotate through oxidation, in *de novo* pyrimidine biosynthesis and donates the electrons to ubiquinone to generate ubiquinol, which is then re-oxidized by ETC complex III[90]. In experimental models of AML, inhibition of DHODH enhances myeloid differentiation[90]. In addition, a decrease in leukemia-initiating cells, as well as increased survival in vivo, have also been observed following the inhibition of DHODH[91]. DHODH inhibition promotes myeloid differentiation by depleting uridine, a precursor of uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). The depletion of uridine diphosphate-GlcNAc decreases O-linked N-acetylglycosylation (O-GlcNAc) of several proteins including Akt, TET family of proteins and c-Myc[91]. The loss of O-GlcNAc modification following DHODH inhibition reduced the stability of c-Myc and thereby promoted differentiation in AML[92]. In addition, DHODH inhibition revealed the dysregulation of several key genes that are involved in a maturation block of AML cells, such as HOXA9, FLT3 and c-Myc[93]. Of note, DHODH inhibition using BAY 2402234 induced acute downregulation of HMGA1, a chromatin remodeler that is implicated in stemness, and would otherwise negatively regulate differentiation[93]. In clear cell renal cell carcinoma, the DHODH inhibitor selectively inhibited tumor growth in GPX4-low tumors, while combined treatment with sulfasalazine, which also induces ferroptosis, synergistically inhibited the growth of GPX4-high tumors[94].

Notably, several clinical trials have reported that brequinar, a DHODH inhibitor, has limited efficacy, likely related to a schedule that did not allow sustained exposure[91,95]. ETC complex III helps in maintaining the oxidation of ubiquinol, which is essential to supporting DHODH activity[96]. Interventions targeting respiratory Complex III would increase the generation of ROS and block the activity of DHODH and pyrimidine biosynthesis[97,98], and could thus be an effective two-pronged strategy involving the exploitation of cell death promoted by ROS[99] and differentiation induced by DHODH inhibition. Alternative oxidase (AOX) can restore DHODH activity and reactivate CoQ redox-cycling following DHODH inhibition[100].

Aspartate biosynthesis is a crucial step in pyrimidine biosynthesis, which requires ETC complex I activity[15]. AML's dependency on ETC complex I has been well-documented. As a result, there has been an increasing interest in targeting ETC complex I and induction chemotherapy together.

TARGETING NICOTINAMIDE METABOLISM

LSCs from relapsed/refractory AML patients are resistant to venetoclax + azacytidine, in contrast to those from *de novo* AML patients, suggesting that they have alternative resistance mechanisms. Jones *et al.* (2020) determined whether LSCs from relapsed/refractory AML patients exhibited altered metabolome profiles compared to those from *de novo* AML; they identified 18 metabolites that were upregulated, including amino acids and nicotinamide[101]. Interestingly, relapsed LSCs exhibited an enhanced rate of NAD+ synthesis driven by the salvage pathway, with no difference in the protein levels of NAMPT, the rate-limiting enzyme responsible for the generation of NAD+ from nicotinamide, suggesting an increase in nicotinamide uptake in relapsed/refractory LSCs[101]. The elevation of nicotinamide metabolism in relapsed LSCs, activates amino acid metabolism and FAO, which in turn drives OXPHOS. An increase in OXPHOS would help LSCs to confer resistance to venetoclax + azacytidine[101].

Unlike *de novo* LSCs, relapsed/refractory LSCs exhibit extensive metabolic plasticity, which enables them to adapt alternative mechanisms for activating OXPHOS, a key mechanism for developing resistance to chemotherapy[101]. Given that therapeutic options are limited for relapsed/refractory AML, the inhibition of NAMPT, the rate-limiting enzyme for NAD+ synthesis, may be an effective approach[101]. Notably, relapsed/refractory LSCs were selectively eradicated by the genetic and pharmacological inhibition of NAMPT, while normal hematopoietic stem/progenitor cells were spared. Of note, in secondary transplantation of relapsed/refractory AML LSCs, in-vivo treatment with APO866, a small molecule inhibitor of NAMPT, compromised the engraftment[101].

TARGETING FATTY ACID METABOLISM

FA catabolism has been implicated in regulating HSC fate (i.e., maintenance *vs.* exhaustion) by providing the required energy to allow the proper execution of asymmetric division, favoring the maintenance and function of the HSC compartment. Loss of the mitochondrial FAO activator peroxisome proliferator-activated receptor δ (PPAR-δ) perturbs HSC maintenance, whereas treatment with PPAR-δ agonists restores HSC maintenance by promoting asymmetric HSC division[102]. PPAR-δ activates FAO in mitochondria. LSCs from *de novo* AML patients drive OXPHOS exclusively using amino acid metabolism. In contrast, LSCs from relapsed/refractory AML patients tend to compensate significantly through increased FA metabolism[79]. Despite the importance of FAs for mitochondrial metabolism in LSCs, aberrant lipid metabolism may also promote cancer cell proliferation by increasing its energy availability, structural lipid-based building blocks, and signaling molecules[103]. Vriens *et al.* demonstrated that cancer cells exhibit extensive plasticity in FA metabolism and can facilitate membrane biosynthesis during proliferation through the FA desaturation pathway[103]. Further investigation is needed to determine whether such metabolic plasticity exists in leukemic cells and if it could be targeted.

Mcl1 is an antiapoptotic protein that is known to interact with proapoptotic proteins in mitochondria. Mcl1 upregulation is common in venetoclax-resistant AML[104]. Interestingly, MCl1 in cancer cells regulates lipid metabolism through a functional gain. This is achieved by binding its α- helix of the BH3 domain to very long-chain acyl-CoA dehydrogenase (VLCAD), a key enzyme of the mitochondrial FAO pathway, through its hydrophobic surface[105]. Thus, MCL1 not only suppresses apoptosis in cancer cells, but also regulates β-oxidation in response to stress. Of note, Mcl-1 is essential for the efficient assembly of F1F0-ATP synthase oligomers and thus is important for maintaining mitochondrial cristae structure and efficient ATP

synthesis[106]. Another study revealed the bifunctional activities of BAD, demonstrating glucokinase regulation by the proapoptotic BH3 domain of BAD[107]. The findings of these studies have opened a new paradigm, revealing the role of Bcl2 family proteins in not only regulating mitochondrial apoptosis but also energy metabolism in cancer cells. It would be interesting to further explore whether Bcl2 family protein mediated regulation of metabolism plays a role in resistance to standard chemotherapies in AML and to further identify downstream druggable non-canonical targets.

Fatty acid metabolism and resistance to therapy

Cellular levels of ceramide are key determinants of response to several chemotherapeutic drugs. Modulation of the ceramide pathway has been implicated in apoptosis induced by daunorubicin in AML cells[108]. Ceramidases convert ceramide to sphingosine. Kao *et al*. found that daunorubicin and cytarabine treatment resulted in the increased expression and activity of enzymes involved in the ceramide pathway, leading to a decrease in ceramide levels and an increase in ceramide 1- and sphingosine 1-phosphate levels. These perturbations induced remodeling of mitochondria in cancer cells and promoted chemoresistance[109]. For instance, FLT3-ITD signaling activation suppresses pro-cell death lipid ceramide generation in AML cells[110]. Intriguingly, LCL-461, a mitochondria-targeted ceramide analog, effectively induced lethal mitophagy in human AML blasts and crenolanib (FLT3-ITD inhibitor)-resistant AML xenografts, suggesting that activation of mitochondrial ceramide synthesis overcomes resistance to FLT3 inhibition[110].

A previous study reported that *de novo* cholesterol synthesis through the mevalonate pathway plays a key role in AML[111]. Recently, using genome-scale metabolic modeling, Karakitsou *et al*. demonstrated that AraC-resistant cells could be targeted by suppressing cholesterol biosynthesis through the inhibition of squalene synthase[112].

Acetyl-CoA is converted to malonyl-CoA by Ac-CoA carboxylases (ACCs). FA synthase (FASN) catalyzes the condensation of malonyl-CoA and Ac-CoA to generate palmitate as the first product of FA synthesis[113]. FASN is significantly elevated in AML blasts compared to healthy granulocytes or CD34+ hematopoietic progenitors at the RNA level. Inhibition of FASN expression on one side accelerated differentiation of APL cells, on the other hand, sensitized ATRA resistant -non-APL AML cells to ATRA, by promoting translocation of transcription factor EB (TFEB) to nucleus and lysosomal biogenesis[114].

Cellular stress directly regulates FA metabolism by affecting the enzymatic activity of Acetyl-CoA carboxylases (ACC), namely ACC1 and ACC2. AMPK inhibits ACC under energy stress and thus activates fat synthesis and catabolism. Nutrient abundance, on the other hand, downregulates the activity of AMPK, and ACC1 and ACC2 are no longer repressed[115]. This suggests that ACCs act as a regulatory node in FA metabolism by sensing nutrient availability and balancing the switch between anabolic and catabolic processes. Of note, ACC1 suppressed growth-promoting activity and promoted the generation of ROS in primary BM cultures. In addition, ACC1 has been shown to promote myeloid differentiation and delay the development of AML in mice[116]. Hydroxylation of ACC2 by prolyl-hydroxylase 3 (PHD3) increases the activity of ACC2, which, in turn, prevents FA utilization[117,118]. PHD3 levels are decreased in AML[119], thus generating a vulnerability due to dependence on FAs, which can make FAO inhibition an attractive strategy to control AML.

Venetoclax + azacytidine-resistant LSCs exhibit increased levels of several metabolites that are responsible for the transport of FAs into mitochondria at baseline, including acetyl-carnitine, iso-butylryl carnitine, and hexanoyl-carnitine, compared to sensitive LSCs[120]. Venetoclax + azacydine treatment results in a reduction in amino acid uptake by LSCs. Enhanced FA transport facilitates FAO and allows LSCs to compensate for

amino acid uptake loss caused by venetoclax + azacytidine treatment, resulting in resistance. However, this resistance can be overcome by the knockdown of very long-chain acyl-CoA dehydrogenase (ACADVL), an enzyme involved in mitochondrial FA beta-oxidation[120], suggesting that FA beta-oxidation plays an important role in conferring resistance to venetoclax + azacytidine treatment in LSCs.

FAs are conjugated to carnitine by CPT1 to facilitate mitochondrial translocation, and both the PPARs and the coactivator-1 of PPARs modulate CPT1A expression[121]. Pharmacological inhibition of CPT1 by etomoxir depleted refractory LSC function[122], and eliminated venetoclax+ azacytidine-resistant LSCs[85]. In addition, CPT1A inhibition by etomoxir induced cell death in AML cells and increased cells' sensitivity to cytarabine[123]. CD36+ LSCs are characterized by an enhanced FAO rate, and disruption of mitochondrial metabolism by targeting CD36-FAO-OXPHOS drives leukemia cells to low OXPHOS and enhances AraC sensitivity[19]. In addition, topoisomerase inhibitor (mitoxantrone)-resistant AML cells showed activation of lipid metabolic pathways. Inhibition of FAO, using etomoxir in these cells, reduced colony formation ability, suggesting that FA metabolism and dependency on OXPHOS are key vulnerabilities, conferring chemoresistance in AML[124].

TUMOR MICROENVIRONMENT-MEDIATED METABOLIC PLASTICITY

Tumor microenvironment-mediated OXPHOS regulation

BMSCs, which are composed of endothelial cells, osteoclasts, osteoblasts, adipocytes, and fibroblasts, form the BM microenvironment. BMSCs aid the survival of AML cells and mediate resistance to therapy. Cell-cell communications in the BM microenvironment play a key role in aiding AML cell survival, disease progression, and therapeutic response through the transfer of biomolecules or cellular organelles, such as mitochondria[125]. Of note, BMSCs supply mitochondria to AML cells in vitro, thus providing them with additional energy[126]. AML cells were observed to enhance their mitochondrial mass up to 14% by mitochondrial uptake from BMSCs; this transfer increased in response to chemotherapy and contributed to resistance by reducing mitochondrial depolarization[126]. AML cells exhibited up to a 1.5-fold increase in mitochondrial ATP production following the uptake of mitochondria from BMSCs, were less prone to depolarization of mitochondria following chemotherapy, and showed improved survival[126]. Of note, transfer of mitochondria to AML cells from BMSCs in the presence of chemotherapeutic agents occurs predominantly through endocytosis and requires cell-cell contacts[12]. The NADPH oxidase, NOX2 generates superoxide in AML cells, which promotes mitochondrial transfer to AML blasts from BMSCs through AML-derived tunneling nanotubes. Interestingly, suppression of NOX2 reduced the transfer of mitochondria to AML blasts and resulted in enhanced cell death and improved survival of AML-bearing mice[126]. AML cells show high OXPHOS levels when cocultured with BMSCs. Hou *et al.* showed that the elevation of OXPHOS levels in AML cells in coculture was dependent on the activation of mitochondrial serine-phosphorylated STAT3 (pS-STAT3)[127]. AML cells in coculture induced the secretion of interleukin-6 from BMSCs, which in turn promoted the activation of total and mitochondrial STAT3 in AML cells, resulting in increased proliferation and chemoresistance[127].

AML cells are known to harbor abnormally high levels of ROS. Mesenchymal stem cells, a component of BMSCs, support the survival of AML cells by influencing several key metabolic pathways and eliminating ROS. BMSCs expressing the intermediate filament protein nestin provide HSC niche function[128]. Forte *et al.* reported that after AML induction, unlike bulk stroma, the nestin-expressing BMSC cell population remains stable in MLL-AF9-driven AML, with no reduction in their number over time[129]. However, these cells undergo changes in their functional behavior to enhance the survival of MLL-AF9 AML cells and promote chemoresistance by promoting TCA cycle and OXPHOS. They also provide a GSH-mediated antioxidant defense, which is critical for ROS mitigation during leukemogenesis and chemotherapy[129].

Forte *et al.* observed a delay in leukemogenesis, following the depletion of Nestin$^+$ BMSCs in primary AML mice[129]. Most importantly, the depletion of Nestin+ BMSCs in chimeric mice reduces AML, but not normal cells[129]. Thus, BMSCs help AML cells switch their energy sources and provide antioxidant defense mechanisms to survive chemotherapy.

Tumor microenvironment mediated regulation of amino acid and pyrimidine metabolism

Induction chemotherapy causes transient stress in BM, which results in the elevation of glutamine, glutamate, and aspartate with a concomitant increase in the expression of several glutamine transporters, such as Slc1a5, Slc38a1, Slc7a5, and Slc7a6, but no change in the enzymes that metabolize glutamine or glutamate, in residual AML cells[130,131]. This suggests that glutamine metabolism in AML cells could be regulated by the tumor microenvironment[130,131]. Interestingly, van Gastel *et al.* showed that glutamine metabolism differed between AML and stromal BM cells, especially LepR+ mesenchymal stromal cells[131]. AML cells took up glutamine to maintain GSH but not to promote the TCA cycle. In contrast, in LepR+ mesenchymal stromal cells, glutamine entered the TCA cycle to produce aspartate[131]. Aspartate is transported to and supports pyrimidine biosynthesis in persistent MLL-AF9 AML cells, thus preventing the metabolic collapse triggered by chemotherapy[130,131]. These findings suggest that the tumor microenvironment plays a major role in chemoresistance, independent of cell-intrinsic signaling/apoptosis alterations. However, it is unclear whether aspartate-rich locations in the BM provide a protective niche, and if specific AML subclones compete for these spots. Further niche-specific studies are warranted to delineate whether mutational subclones compete for specific hotspots.

Tumor microenvironment mediated regulation of fatty acid metabolism

Accumulating evidence supports the existence of metabolic symbiosis and bidirectional dialogues between leukemic cells and the BM adipose tissue niche, which provides diverse sources of lipids, such as FAs, cholesterol, and eicosanoids, to resident leukemic cells. Leukemic cells meet their energy needs by using FAs to fuel β-oxidation, which eventually supports anabolism[33,132]. When FAO was inhibited pharmacologically with etomoxir or ranolazine, AML cells were sensitized to apoptosis induction by ABT-737, and proliferation was inhibited in the setting of coculture with BMSCs[123]. Interestingly, a recent report described two forms of LSCs, distinguished by differences in their expression of CD36 and in their metabolism and cell cycle status. LSCs expressing CD36 (CD36+ LSCs) are often localized to gonadal adipose tissue (GAT) which is rich in fatty acids, indicating a tropism rich for microenvironments rich in FAs[132]. Specifically, these GAT-resident LSCs are more resistant to conventional chemotherapy because they exist in a quiescent cell cycle state. In the context of these findings, leukemic cells co-opt adipose tissue to create a microenvironment to support leukemia growth and provide a drug-resistant niche[132]. Further, leukemic burden in GAT for the MLL-AF9 model was considerably lower than that in BM, indicating that localization to GAT may be dependent on the specific oncogenes that drive the malignant transformation process[132]. An interesting question for future investigations would be to understand the role of FA metabolism in conferring drug resistance to LSCs in a niche-specific BM microenvironment.

ROLE OF EXTRACELLULAR VESICLES IN CONFERRING RESISTANCE BY MEDIATING COMMUNICATION BETWEEN LEUKEMIC CELLS AND MICROENVIRONMENT TO ALTER METABOLISM

AML cells alter the BM by releasing exosomes, which promote the proliferation and survival of leukemic cells and suppress normal hematopoiesis. In a study by Javidi-Sharifi *et al.*, the expression of FGF2 and its receptor, FGFR1, were both increased in several stromal cell lines and primary AML stroma. This heightened FGF2/FGFR1 signaling augmented greater secretion of exosomes[133]. Huang *et al.* (2021), studied the impact of inhibition of small extracellular vesicle (SEV) secretion from various sources on the

progression of MLL-AF9 induced AML, as well as normal hematopoiesis[134]. A significant delay in AML progression occurred when SEV secretion from endothelial cells was inhibited, but not that from perivascular cells, megakaryocytes, or spleen stromal cells[134]. SEVs derived from endothelial cells contained high levels of Angiopoietin-like protein 2 (ANGPTL2) protein, which binds to the leukocyte immunoglobulin-like receptor B2 (LILRB2 receptor) and accelerates leukemia progression[134]. Moreover, Vacuolar protein sorting-associated protein 33B (VPS33B) governed the release of ANGPTL2-SEVs from endothelial cells and this release of ANGPTL2-SEVs was required for primary human AML cell maintenance. These findings demonstrate that SEVs have a role in cancer development and suggest that targeting the release of ANGPTL2-SEVs from endothelial cells would be an effective strategy in the treatment of some types of AML[134]. While IO-108, an antibody targeting LILRB2, is in Phase 1 clinical development for solid tumors[135]; LILRB4 antibody (IO-202) is in phase 1 cohort expansion clinical trial [NCT0437243] in combination with azacitidine and venetoclax, for the treatment of AML and likely to be effective in monocytic AML[136,137].

Cytarabine and decitabine treatment resulted in significantly higher intracellular levels of cholesterol and HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), the rate-limiting enzyme in the cholesterol synthesizing mevalonate pathway, in cultured AML and non-malignant cells. Concomitantly, these cells produced higher levels of SEVs[138]. Interestingly, there was an increase in HMGCR levels in SEVs in response to chemotherapy. These HMGCR+ SEVs promoted the growth of AML cells by upregulating intracellular cholesterol levels. This chemotherapy-induced enhancement of SEV secretion in AML cells was prevented by HMGCR inhibition[138]. Thus, HMGCR blockade could potentially provide a therapeutic alternative in AML by inhibiting cholesterol-driven chemoresistance caused by SEV signaling[138]. Another study reported the secretion of exosomes containing vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) by AML cells. These exosomes induced glycolysis in human umbilical vein endothelial cells (HUVECs), which resulted in vascular remodeling and chemoresistance[139].

BM stromal cells exposed to AML-derived exosomes exhibit decreased expression of genes supporting normal hematopoiesis (CXCL12, KITL, IL-7, IGF1) and osteogenesis (OCN, Col1A1, IGF1) and increased expression of genes supporting AML growth (DKK1, IL-6, CCL3)[140]. When BM is preconditioned with AML-derived exosomes, AML cell engraftment and growth are significantly accelerated, whereas disruption of exosome secretion in AML cells significantly reduced these cells' ability to engraft and grow *in vivo*[140]. In coculture, serum and leukemic cells from patients with NPM1-mutated AML impair CD8+ T cells' immune function[141]. Through SEVs, leukemic cells secrete miR-19a-3p into the tumor microenvironment. This process was mediated by the NPM1-mutated protein/CCCTC-binding factor (CTCF)/poly (A)-binding protein cytoplasmic 1 (PABPC1). MiR-19a-3p released from SEV was found to be internalized by CD8+ T cells, where it inhibited creatine import by repressing the expression of a transporter solute-carrier family 6 member 8 (SLC6A8)[141]. A reduction in creatine levels lowered ATP production and impaired CD8+ T cell immune function, which caused leukemic cells to escape the immune system[141]. Interestingly, the exosomes generated by fully engrafted AML PDX mice were similar to exosomes isolated from the plasma of the patients who had donated the cells for engraftment. These exosomes carried human proteins and leukemia-associated antigens, confirming their origin from AML patients[142].

AML utilizes SEV miRNAs to target the mTOR pathway which suppresses protein synthesis in the LT-HSC to elicit their quiescence[143]. While LT-HSCs are functional after the recovery, their DNA is damaged for a long time. Hematopoietic stem and progenitor cells (HSPCs) are generally susceptible to SEV entry and use the mTOR pathway, but HSCs selectively enter quiescence because of their unique sensitivity to protein synthesis disruption[143].

TECHNIQUES TO ASSESS MITOCHONDRIAL FUNCTION AND METABOLISM

A tightly coordinated flux of metabolites occurs in mitochondria, integrating bioenergetics, redox homeostasis, and anabolic metabolism. By assessing mitochondrial function, researchers have gained a better understanding of metabolism in cellular physiology, disease pathology, and etiology. The reduction of oxygen into water is essential to oxidative phosphorylation and measuring oxygen consumption is therefore one way to assess mitochondrial function[144]. The mitostress test using a high-throughput real-time mode approach in Agilent Seahorse XF analyzers is the best assay for measuring basal cell respiratory rate, ATP production, proton leak, maximum respiration, and spare respiratory capacity in an intact cell[145]. This single assay yields a wide range of parameters, providing insight into mitochondrial dysfunction and reveals the functional differences between cell types, cellular metabolic state (quiescent versus active), effect of drug candidates, impact of genetic modifications, or biochemical interactions[146].

Mitochondria are known to generate approximately 90% of cellular reactive oxygen species (ROS)[147]. As a result of excessive production of ROS and/or decreased antioxidant defense activity, mitochondrial reactive oxygen species (mtROS) accumulate, causing oxidative stress (OS). It leads to oxidative damage that affects several cellular components, including lipids, DNA, and proteins[147]. To detect mitochondrial superoxide selectively, MitoSOX Red and Green are used, which are fluorogenic reagents designed for highly selective detection of superoxide in the mitochondria of viable cells[148]. A notable feature of these probes is that they are readily and specifically oxidized by superoxide alone and not by other ROS or reactive nitrogen species (RNS) generating systems at optimum concentration. However, the user should be aware of the cytotoxic or mitotoxic effect of these probes and their potential impact on mitochondrial morphology at higher concentrations[149]. Furthermore, oxidation of these probes is inhibited by superoxide dismutase. Small, cell-permeant dyes such as TMRM (tetramethylrhodamine, methyl ester) and TMRE (tetramethylrhodamine, ethyl ester) accumulate in active mitochondria and may be used to quantify mitochondrial membrane potential[150]. They appear bright in healthy cells with functioning mitochondria, but are dimmed or undetectable upon loss of mitochondrial membrane potential[151].

Metabolome consists of all the molecules with low molecular weight (metabolites) within a cell, tissue or organism. Analyzing the metabolites within a cell, tissue or organism following a genetic change or physiological stimulus is known as metabolomics[152]. The metabolomics approach can be divided into two distinct types: untargeted metabolomics and targeted metabolomics. Targeted approaches focus on identifying and quantifying a small number of known metabolites, such as those found in clinical analyses. In untargeted approaches or hypothesis-generating approaches, data are obtained for as many species as possible, metabolites are annotated, and metabolic changes are reviewed regardless of whether they are known or unknown[153]. However, measuring metabolite concentrations by metabolomics only tells half the story. The accumulation of metabolites can be caused not only by increased production, but also by decreased consumption. The metabolic flux, which can be quantified as material flow per unit time, is equally important for understanding pathway activity[153].

Unlike metabolites, fluxes are not physical entities that can be detected by mass spectrometry. But they can be inferred by using isotope tracers. Radioactive tracer studies laid the groundwork for modern metabolism research[154]. Today, similar studies can be performed using stable non-radioactive isotope tracers, which can be tracked quantitatively and broadly using MS or NMR[155]. Furthermore, an approach called metabolic activity screening integrates the metabolomics data with metabolic pathways and systems biology data, including proteomics and transcriptomics, in order to identify endogenous metabolites that may alter phenotypic characteristics and functionality[156].

CONCLUDING REMARKS

Nine therapeutic agents for AML have been developed and approved since 2017. Treatment improvements have increased the overall survival rate of AML patients, from 13% to 55% in those aged younger than 60 years and 8% to 17% in patients older than 60 years[157]. Further improvements in our understanding of the disease would further improve outcomes. The genetic makeup of AML, as well as its metabolic requirements for survival and proliferation, are heterogeneous. Diverse types of metabolic vulnerabilities, including dependence on OXPHOS, amino acid metabolism, nucleotide metabolism, and fatty acid metabolism, were reported. In addition, metabolic changes occurring in the tumor microenvironment have also been used as therapeutic targets. However, a thorough understanding of biomarkers of response to these therapies and mechanisms of resistance and combinatorial therapies would pave the way for better clinical management of AML. Understanding how the genetic architecture works with the tumor microenvironment is critical to uncovering new vulnerabilities. In addition, a thorough understanding of the interactions between different cell populations within the BM microenvironment in response to transient stress due to therapy which leads to changes in nutrient availability and metabolic adaptations, will increase the chances of a true cure.

LSCs have metabolically distinct subpopulations and relapsed/refractory LSCs switch to alternative metabolic pathways to fuel OXPHOS, rendering them therapeutically resistant to conventional treatments. Developing treatment strategies that target these metabolic pathways is key to eliminating relapsed/refractory LSC populations and improving treatment outcomes.

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Cancer Drug Resistance

Editorial

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New mechanisms of multidrug resistance: an introduction to the *Cancer Drug Resistance* special collection

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Abstract

Cancer Drug Resistance publishes contributions to understanding the biology and consequences of mechanisms that interfere with successful treatment of cancer. Since virtually all patients who die of metastatic cancer have multidrug-resistant tumors, improved treatment will require an understanding of the mechanisms of resistance to design therapies that circumvent these mechanisms, exploit these mechanisms, or inactivate these multidrug resistance mechanisms. One example of a resistance mechanism is the expression of ATP-binding cassette efflux pumps, but unfortunately, inhibition of these transporters has not proved to be the solution to overcome multidrug resistance in cancer. Other mechanisms that confer multidrug resistance, and the confluence of multiple different mechanisms (multifactorial multidrug resistance) have been identified, and it is the goal of this Special Collection to expand this catalog of potential multidrug resistance mechanisms, to explore novel ways to overcome resistance, and to present thoughtful reviews on the problem of multidrug resistance in cancer.

Keywords: Cancer drug resistance, multidrug resistance, ABC transporters, novel mechanisms of drug resistance

WHY MULTIDRUG RESISTANCE IN CANCER REMAINS AN UNSOLVED PROBLEM

The molecular understanding of the factors that contribute to the growth, invasiveness, and metastasis of cancer has led to an explosion of new treatments for cancer, including the development of targeted

chemotherapeutics. Unfortunately, although there have been clear improvements in early diagnosis and treatment of cancer, leading to improved prognosis and duration of survival, the patient with a metastatic solid tumor or many kinds of hematologic cancer will not be cured and will eventually succumb to their disease. This leads inevitably to the conclusion that curative treatments for cancer will require a much better understanding of the many mechanisms that result in resistance to treatment, especially multidrug resistance to chemotherapy.

A summary of the various means by which cancer cells might resist chemotherapy was published in 2016[1]. These categories are still relevant today and include: (1) For targeted drugs, alterations by mutation in the targets or activation of alternative growth promoting pathways; (2) Changes in cell-based pharmacology, including drug efflux pumps, altered uptake systems, altered metabolism, sequestration and altered trafficking of drugs, *etc*.; (3) Epigenetic alterations in differentiation pathways, such as Epithelial to Mesenchymal Transition (EMT), altered transcription patterns, cell survival adaptations, loss of cell death pathways and stem cell-like states; and (4) Alterations in local tumor physiology and substratum, including other tumor-associated cells, changes in physical properties of the substratum, and altered blood supply. All of these potential resistance mechanisms can be facilitated by many of the known hallmarks of cancer, such as heterogeneity and genetic and epigenetic plasticity, which now include several characteristics such as resistance to apoptosis and avoidance of immune destruction, which help confer resistance to anti-cancer drugs[2].

Recent reviews and original research articles have identified some novel mechanisms of resistance that fall into these categories but were unexplored until recently. These include the concepts of dormancy[3], persistence[4], metabolic plasticity[5], the role of tumor-associated fibroblasts[6], the integrated stress response[7], the role of long noncoding RNAs (lncRNAs)[8], microvesicles[9], and transcriptional variability[10]. To no one's surprise, the microbiome has turned out to affect response to chemotherapy[11] and the constituents and physical properties of the extracellular matrix are also determinative[12]. In addition, the use of machine deep learning to analyze the response of human cancer cells to therapy has been proposed as a new way to predict the success of chemotherapy[13]. Ultimately, it might turn out that as cancers evolve and become more drug-resistant, they begin to morph into cells that are more like non-cancer cells in their metabolic pathways and physiology (and hence relatively resistant to chemotherapy) while still maintaining their growth, invasiveness, and metastatic phenotypes.

There are approximately 10,000 articles listed in PubMed that relate to mechanisms of drug resistance in cancer, yet we are still unable to identify the key features that result in the terminal multidrug resistance of many cancers. This conundrum was pointed out over a decade ago by Piet Borst in a prescient commentary in which he coined the term "pan-resistance" to describe this terminal resistance state[14]. In this article, he explores a variety of explanations for this phenomenon and concludes that much more work is needed, particularly in the design of relevant animal models to study this phenomenon. We agree that much more information is needed and encourage the submission of articles that explore in more detail the basis of known molecular mechanisms of resistance, identify novel mechanisms of resistance, novel model systems, novel methods of analysis, and novel testable hypotheses.

It is worth considering the ATP-binding cassette (ABC) efflux transporters as a case in point. A substantial part of the existing literature on multidrug resistance is devoted to papers describing new inhibitors of P-glycoprotein (ABCB1) and the ABC transporters ABCG2 (BCRP) and the ABCC family, especially ABCC1 (MRP1). Although these transporters do frequently appear as the cause of multidrug resistance in

tissue culture models, and even animal models of multidrug resistance[15,16], their major contribution to drug resistance appears to be related to their contributions to ADMET (absorption, distribution, metabolism, excretion, and toxicity) for anti-cancer drugs and many other drugs, and to their barrier functions, particularly the blood-brain barrier where their expression in capillary endothelial cells in the brain prevents brain accumulation of a high percentage of drugs in common use, including many chemotherapeutics[17]. There is a clear role for ABC transporters in some cases of drug resistance in the clinic (Robey *et al.*[18]), and new drugs targeted to kill cancer cells frequently turn out to be substrates for ABC transporters[19]. However, more often than not, even for drugs known to be substrates for P-glycoprotein or ABCG2 efflux pumps, other mechanisms of resistance supervene. The consideration here for the cancer cell is what is the most efficient way to survive killing by anti-cancer drugs, and the use of ABC transporters is quite energy dependent.

In another very thoughtful commentary, Borst warns against expansive and inaccurate claims made about the clinical significance of expression of the ABC transporters in tumor cells[20]. There is ample reason to study the complex mechanism of action, substrate and inhibitor specificity, physiological function, and pharmacologic impact of the ABC transporters, but claiming that P-glycoprotein (and even ABCG2 and ABCC1) is "responsible for multidrug resistance in cancer" and that there are no adequate inhibitors can be misleading without additional data implicating these transporters in specific cancers treated with specific drugs. It is our hope that articles submitted to the Special Collection of *Cancer Drug Resistance* will shed new light on the role of the ABC transporters in multidrug resistance, but not focus on another new inhibitor unless it is truly miraculous!

SOME QUESTIONS THAT STILL NEED TO BE ANSWERED ABOUT CANCER MULTIDRUG RESISTANCE

Although not intended to be a complete list of challenges remaining in the field of multidrug resistance, there are still quite a few basic questions that need to be more clearly formulated and answered in the literature and would be excellent subjects for papers submitted to this *Cancer Drug Resistance* Special Collection:

(1) Does resistance to a specific chemotherapy protocol reside among the pre-treatment population of cells, and is selected by the therapy, or does it arise through genetic or epigenetic changes in cancer during the course of therapy? This question harkens back to the 1969 Nobel Prize winning work of Luria and Delbruck[21] that demonstrated using a fluctuation analysis that mutations to resistance pre-existed in the pre-treatment population of bacteria. Similar studies in cultured mammalian cells have confirmed these conclusions, and more recent studies on drug resistance in acute myeloid leukemia (AML), a disease where refractory cells often express ABC transporters[22,23] suggest that most resistant subclones that arise were present prior to therapy[24]. Similar conclusions were reached using melanoma cells and a lineage mapping approach known as FateMap[25]. However, given that most chemotherapy is mutagenic, and that there are quite a few robust transcriptional and epigenetic responses to toxic therapy, it seems unlikely that all cancers will become resistant based on the intrinsic resistance of pre-treatment subclones. This is not an entirely academic question, since if pre-existing mechanisms of resistance can be detected, this would help guide chemotherapy. If resistance occurs post-treatment, then careful monitoring of the tumor population, as by circulating cancer cells or DNA, becomes essential unless these mechanisms turn out to be universal.

(2) Much of the progress in understanding multidrug resistance to date has been based on the application of new technologies. What new technologies should be used to catalog and determine the clinical significance of potential mechanisms of drug resistance? One obvious example is the use of CRISPR-based selections to

determine whether increased or decreased expression of specific genes enhances cell survival or leads to drug resistance[26]. The downside of this approach is that it is limited to testing one gene at a time, but the advantage is that there is an immediate read-out of individual genes contributing to resistance without the need to identify a gene or genes whose expression or function is altered in tissue culture cells selected for resistance to a specific drug. Another technology that should reveal important information about pre-existing *vs*. induced resistance is lineage tracing, which allows the tracking of individual cells during the drug-selection process[27]. The application of both of these approaches to model systems that more closely mimic the growth of human cancers will be an important advance.

(3) Can we develop more tractable model systems to study drug resistance? It is well-established that cultured cancer cells may bear little or no resemblance phenotypically and in terms of gene expression patterns to cells derived directly from cancers[28]. Although some characteristics of the tumor of origin may be obtained, the complex networks that confer a selective advantage under adverse conditions are likely altered in cultured cells. So, a variety of different approaches have been taken to study cancer in the laboratory, including genetically engineered mouse models, primary tumor xenografts, usually in mice but more recently in other easier-to-study models such as zebrafish[29], and cancer cell organoids[30]. Very few of these models have been employed to study drug resistance, and more detailed studies of the evolution of drug resistance resulting in terminal pan-drug resistance, utilizing new, more informative technologies, would be welcome.

(4) Why do many cancers, after months or years of therapy and periods of dormancy, emerge, grow, and metastasize in a state that is entirely resistant to therapy? This is a problem in clinical research, requiring detailed timelines of cancer evolution enabled by improved molecular imaging, study of circulating cancer cells and DNA, and either repeat biopsies or rapid autopsies to allow transcriptional profiling of terminal cancers. Very few of these studies have been undertaken, but investment of the time and resources needed to do these studies would yield huge returns in knowledge and improved treatment.

CONCLUSIONS

Cancer is a difficult adversary. The investment in new, more targeted therapeutics has resulted in improved response, but multidrug resistance remains the major barrier to curative treatment of cancer with chemotherapeutics. New advances in technologies to detect drug resistance genes and model systems in which to study the evolution of cancer drug resistance should accelerate discovery in this area. *Cancer Drug Resistance* welcomes manuscripts that address the questions outlined in this Editorial as well as many others that are relevant and give new insights into the problem of multidrug resistance in cancer.

DECLARATIONS

Authors' contributions

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Consent for publication

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Cancer Drug Resistance

Original Article

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Resistance of breast cancer cells to paclitaxel is associated with low expressions of miRNA-186 and miRNA-7

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Abstract

Aim: Neo-adjuvant chemotherapy is a common approach for the complex treatment of breast cancer (BC) and paclitaxel (PTX) is frequently included in the therapeutic regimen. However, the effect of PTX-based treatment is hard to predict precisely based on routinely used markers. As microRNAs are considered a new promising class of biomarkers, the link between miRNA expression and PTX resistance of BC cells needs to be well investigated. This study aimed at the identification of miRNAs associated with responses of BC cells to PTX.

Methods: Intrinsic PTX sensitivity and miRNA profiling were assayed in five BC cell lines to identify candidate miRNAs. Selected miRNA (*n.* 15) expressions were analyzed by real-time-quantitative polymerase chain reaction (RT-qPCR) in BC tissue samples (*n.* 31) obtained from a diagnostic biopsy. Results were analyzed in the context of the effect of two cycles of PTX and the effect of the completed scheme of neoadjuvant therapy. The study's design facilitated the evaluation of the effect of PTX on cells and the identification of features of the microRNA expression profiles associated exclusively with sensitivity to this drug.

Results: miR-186 and miR-7 expression in BC tissues was higher in patients with better outcomes of PTX-based neoadjuvant therapy.

Conclusion: High expressions of miR-186 and miR-7 are associated with good response to PTX, whereas their low expressions may be associated with resistance to PTX in BC, indicating the possibility of developing innovative test systems for the prediction of the PTX response, which can be used before the start of neo-adjuvant chemotherapy for BC.

Keywords: Breast cancer, paclitaxel, resistance, neoadjuvant therapy, miRNA, miR-186, miR-7

INTRODUCTION

Breast cancer (BC) is a common oncological disease. According to the International Agency for Research on Cancer, more than 2.2 million women were diagnosed with BC in 2020[1]. Despite the constant progress in the development of new diagnostic and therapeutic approaches, BC poses a significant social burden across countries with different economies and healthcare systems. One of the most intriguing problems encountered in the treatment of BC is the disease's extreme heterogeneity[2], which makes it difficult to choose the optimal approach for systemic therapy. Different molecular markers including sex hormone receptors (ER, PR), receptor of epidermal growth factors (HER2), markers of active proliferation (Ki-67, survivin, NGAL), and metastatic potency (MMP9, SK1, DcR3, CIX2, EZH2) are used currently in clinical practice to evaluate tumor properties and define the optimal therapeutic approaches. Several new tests and classifiers have been developed recently, although not approved so far, including PAM50[3], EndoPredict[4], Oncotype DX, Breast Recurrence Score, EndoPredict, Prosigna[5], and others. However, the selection of BC chemotherapy regimen in clinical practice is still based on the short list of tumor characteristics such as histology, grade, stage, and IHC status of hormone receptors (HR), HER2, and Ki-67[6]. These characteristics are far from exhaustive and their complex evaluation does not allow prediction of the inherent sensitivity of BC cells to specific cytostatic agents.

Neoadjuvant therapy (NAT) is integral to the treatment of patients with high-risk breast tumors, large breast tumors, and locally advanced tumors, including those who are initially ineligible for surgery. Several schemes of NAT are approved for early-stage BC of various biological types[7]. For instance, neoadjuvant chemotherapy of HER2-negative BC may include different combinations of cytostatic agents such as anthracyclines, cyclophosphamide, and taxanes[7] that can be administered in different sequential orders[8]. These drugs have varied mechanisms of action-anthracyclines intercalate between DNA base pairs and block essential processes of DNA transcription and replication[9]; cyclophosphamide is an alkylating agent forming intrastrand and interstrand DNA cross-links, thereby affecting DNA function[10], whereas taxanes stabilize microtubules and affect the dynamic of the mitotic spindle[11]. In the background of different mechanisms of action of these drugs, differential intrinsic sensitivity of an individual tumor to each of these is expected. Initiating NAT with presumably the most effective drug or their combination would provide the best response; however, new molecular markers are required to personalize the selection of optimal therapeutic scheme from a list of validated agents.

Paclitaxel (PTX) suppresses the polymerization dynamic of microtubules, resulting in mitotic arrests. Resistance to PTX can be mediated by general mechanisms (such as overexpression of the efflux drug proteins) or is associated with molecular mechanisms of the mitotic process, including tubulin isoforms ratio (TUBB3 overexpression), microtubule-associated proteins (Tau, MAP4) or spindle assembly checkpoint protein (Mad2, BubR1, Aurora A) functionality[12,13]. Complex analysis of these molecules may be helpful to predict the effect of PTX. Among the various molecular markers of multidrug resistance or PTX resistance, small regulatory RNAs (microRNAs) appear to be a promising class[14]. MicroRNAs or

miRNAs are involved in the control of all cellular functions, and some of them directly regulate the expression of proteins mediating the response of BC cells to PTX. For instance, miR-125b confers PTX resistance to BC cells through suppression of pro-apoptotic Bcl-2 antagonist Killer 1 (Bak1) expression[15]. miR-451 is involved in the resistance of BC cells to PTX by targeting key regulators of cell survival and apoptotic machine 14-3-3ζ proteins (YWHAZ)[16]. miR-152 suppresses endothelial PAS domain-containing protein 1 (EPAS1), thereby enhancing the apoptosis rate and susceptibility of BC cells to PTX[17]. miR-155 contributes to PTX resistance of BC cells through tumor protein p53 inducible nuclear protein 1 (TP53INP1)[18]. miR-16 sensitizes BC cells to PTX through the suppression of IKBKB expression[19]. miR-520h stimulates PTX resistance by targeting the OTUD3-PTEN axis in BC cells[20]. Scientific data indicate the involvement of specific miRNA molecules in controlling PTX resistance. However, only a limited number of systemic investigations of the miRNA-mediated response of BC to PTX have been performed so far and the results of these studies are hardly comparable. Uhr *et al.* investigated the expression of 411 miRNAs in the context of sensitivity to 34 drugs (including PTX) in 36 BC cell[21]. This large-scale experimental study showed an association between PTX sensitivity and miR-187, miR-106a, and miR-556 expression; however, these results were relevant to *in vitro* cultured cells only and were not evaluated in BC tissues. In contrast, Wu *et al.* investigated the network of transcription factor-miRNA-mRNA in samples of BC tissues (*n.* 50) before and after treatment with PTX[22]. They observed an association between the effect of PTX and the expressional profile shift of several miRNAs (miR-508, miR-4445, miR-3545, miR-1911, miR-584, miR-4782, and miR-219). These exciting computational data require experimental validation.

In our study, we attempted to combine and take advantage of both experimental and observational approaches. First, potential markers of BC cell resistance to PTX were identified by combined analysis of miRNA expression profiles and intrinsic PTX sensitivity of five BC cell lines. Second, the expressions of selected miRNAs were analyzed in tumor tissues of patients with BC and their association with the effect of PTX-based NAT was verified. As the mechanism of PTX action is not related to the HR/HER2 status, patients with different BC subtypes were included in the study. The NAT of all patients was started with PTX, which allowed us to attribute the effect of the first two cycles to PTX sensitivity only. With this approach, we could identify miRNAs that are most likely associated with the phenomenon of inherent resistance of BC cells to PTX.

METHODS

BC cell lines

Cell lines were obtained from the Institute of Cytology (Russian Academy of Sciences). MCF7, BT20, MDAMB231, and MDAMB453 are widely used BC tissue-derived cell lines. The HBL-100 line was obtained from primary cultures of cells derived from an early lactation sample of human milk[23]; however, following studies revealed the presence of Y chromosome and malignant characteristics of these cells[24,25]. Small tandem repeat (STR) profiling was performed as a commercial service (InLab-genetics.ru) to confirm the identity of all used cell cultures [Supplementary Appendix 1-5]. All cell lines were cultured in RPMI 1,640 medium (PanEco, Moscow, RF) supplemented with 10% heat-inactivated fetal bovine serum (Biosera, Nuaille, France) and 1% penicillin-streptomycin (PanEco, Moscow, RF) at standard conditions.

Immunohistochemistry

Cells were cultivated on 4-chamber slides till they reached sufficient confluence. They were washed with PBS, fixed with 4% paraformaldehyde, permeabilized (for nuclear receptors assay) with 0.1% Triton X-100, blocked with 2% BSA, incubated with rabbit antibodies against ER, PR, or HER2 (Ventana Medical System Inc., Tucson, USA, cat. No. 790-4,325, 790-429, 6,790-4,493) and stained following the protocol specified in the detection kits (Ventana Medical System Inc., Tucson, USA).

Flow cytometry

Cells in suspension (1 million cells/250 μL) were treated with BD Cytofix/Cytoperm Kit (BD Bioscience, Heidelberg, Germany), washed with PBS, incubated with antibodies against ER, PR, or HER2 (Ventana Medical System Inc., Tucson, USA, cat. No. 790-4,325, 790-429, 6,790-4,493), washed again and stained with secondary anti-rabbit FITC-labeled antibodies (Abcam, Cambridge, UK, cat. No. AB6717). Analysis was performed using a CytoFLEX cytometer (Beckman Coulter, Brea, USA).

PTX toxicity and cell viability assays

Cells were seeded into 96-well plates and incubated till they reached 50% confluence. PTX (Sigma-Aldrich, St Louis, USA) was dissolved in DMSO to make the stock concentration of 2 mm, and working concentrations (200-1 nM) of the drug in a complete medium were prepared by serial dilution. PTX was applied to the cells at different concentrations by medium replacement. Control cells were treated with 0.01% DMSO in a complete medium, which corresponded to the amount of DMSO in PTX-treated cells at a maximum concentration (200 nM). Cells were incubated for 72 h and cell viability was assayed with the WST kit (Abcam, Cambridge, UK) following the manufacturer's protocol. The absorbance was measured at 450 nm using a multi-plate reader, Varioscan LUX (Thermo Fisher Scientific, Wolfteam, USA). Each measurement was done in triplicate and the results reflected the average value. The cell viability was expressed as a percentage of viable untreated cells (Mean ODsample/Mean ODcontrol × 100).

Patients

The study design was approved by the Ethics Committee of Petrov's NMRC of oncology 04.02.2021. All patients involved in the study signed the informed consent form. The diagnostic evaluation included whole breast ultrasound (US) investigation of the tumor and core biopsy; the NAT was started with PTX. The effect of therapy was evaluated after the first two cycles. The majority of patients (*n.* 18) included in the study received PTX monotherapy. In these cases, the expected effect was achieved, which allowed the operation to be performed. In some patients, the clinical effect of PTX was worse and after 6 (*n.* 4) or 12 (*n.* 9) cycles, PTX was replaced by a combination of doxorubicin and cyclophosphamide (AC). Radiation therapy before surgery was not applied in any of the patients. All clinical data obtained were depersonalized before analysis.

RNA isolation

Total RNA was extracted from the cell pellets containing one million cells with miRNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol to analyze miRNA from BC cells. To analyze miRNA from BC tissues, material obtained from diagnostic biopsy was used. After morphological and IHC confirmation of the diagnosis, formalin-fixed, paraffin-emdedded (FFPE) tissue samples were sectioned, deparaffinized by incubation with mineral oil and ethanol, and treated with proteinase K (Sileks Ltd., Moscow, RF). The digested samples were centrifuged at 10,000 g for 10 min and the supernatant was mixed with lysis buffer (guanidine thiocyanate, 2 m; sodium acetate pH 4 and 0.6 m; octanic acid, 0.5%, and 2% 2-mercaptoethanol), incubated for 3 min and centrifuged at 10,000 g for 3 min. The supernatant was mixed with 400 μL of 95% ethanol and transferred into a silica spin column (BioSilica Ltd., Moscow, RF). The columns were washed twice with washing buffer and centrifuged to remove excess liquid. RNA was eluted with 120 μL RNA elution solution (EDTA pH 9,510 mm). The RNA quality and concentration were assayed with a Qubit fluorimeter (Thermo Fisher Scientific, Wolfteam, USA).

miRNA analysis

To profile miRNA in the culture of BC cells, the cDNA library was prepared using the Illumina TruSeq Small RNA library preparation Kit following the manufacturer's instructions. The libraries were sequenced on the Illumina HiSeq 3,000 platform, and sequencing reads were aligned with the STAR[26] and normalized

using DESeq2[27]. To assay the expression of selected miRNAs in BC tissue samples, reverse transcription was performed with two-tailed primer[28] followed by PCR using ALMIR-miRNA Assay Kits and protocols (Algimed Techno, Minsk, Belarus) and Real-Time CFX96 Touch instrument (Bio-Rad, Hercules, USA).

Statistical treatment and software

Statistical analyses were performed and images were drawn using Microsoft Excel 2016 (Microsoft Corporation, USA), CytExpert Software 3 (Beckman Coulter, Inc., USA), Quest Graph™ IC50 Calculator (AAT Bioquest, Inc., USA), OriginPro 9.1 software (OriginLab Corporation, USA), GraphPad Prism software (GraphPad Software Inc., USA). To evaluate the statistical significance of the correlation between two parameters, the Pearson correlation coefficient (Pearson's r) was estimated. To evaluate the statistical significance of the difference between the two groups, the non-parametric Mann-Whitney test was applied.

RESULTS

Study design

To select miRNAs potentially associated with the response of BC cells to PTX, five cell lines were explicitly characterized for morphology, HR/HER2 status, PTX sensitivity, and miRNA profiling. This step allowed us to select 15 candidate molecules whose expression level was correlated with PTX sensitivity. The expression of these miRNAs was then assayed by RT-PCR in BC tissues from patients (*n.* 31) with different effects of PTX-based therapy [Figure 1].

BC cell line characteristics

To model the diversity of BC, we used five different BC cell cultures (MCF7, BT20, MDAMB231, MDAMB453, and HBL100). All cell cultures included in the study were established and characterized explicitly[29-33]. Given that many passages can alter the characteristics of the cells, we evaluated their STR profiles and confirmed their identity [Supplementary Appendix 1-5]. The cells exhibited different morphologies [Supplementary Figure 1], thus reflecting the natural diversity of BC. Next, we analyzed the expression of steroid hormone receptors (estrogen receptor, ERα; progesterone receptor, PG) and receptor of epidermal growth factor (HER2). Expression of ER and PR evaluated by IHC using antibodies routinely used for BC tissue staining was almost non-detectable in all cell lines (not shown). Flow cytometry is an additional approach to validate ER, PR and HER2 marker expression[34]. Herein, flow cytometry was found to be more sensitive than IHC. Results are presented in Table 1.

Results are presented as a percentage of positive cells estimated as (positive cells · 100)/all assayed cells using CytEXPERT software. The difference in the expression of each marker within the five cell cultures is additionally demonstrated by color, while the maximum expression level is indicated by a completely shaded area, and the lower expression level is indicated by a proportionally smaller colored area.

We confirmed the ER- and PR-positive status of MCF7 cell line using flow cytometry. Moreover, other cell lines also showed ER and PR staining, but the signals were weaker than that of MCF7. This unexpected result was obtained with MDAMB453 cells, which were ER- and PR-positive as shown in Figure 2A (color legend are the same as in Figure 2) and confirmed by IHC [Figure 2B].

These observations contradict previously published data[32,33] and may reflect the modification of cell phenotype or show the high sensitivity of flow cytometry.

PTX sensitivity of BC cells *in vitro*

All five cell lines were treated with PTX with different concentrations for 72 h and WST test was used to evaluate cell viability. IC50 values were calculated using the Quest Graph™ IC50 Calculator [Figure 3].

Table 1. Expression of ER, PR and HER2 in BC cell lines assayed by flow cytometry

REC\CELLS	MD A321	B T 20	MC F7	MD A435	H B L100
ER	1.78%	1.25%	4.64%	2.14%	1.39%
PR	1.60%	1.37%	3.52%	6.98%	1.71%
HER2	0.55%	2.41%	1.81%	76.25%	1.33%

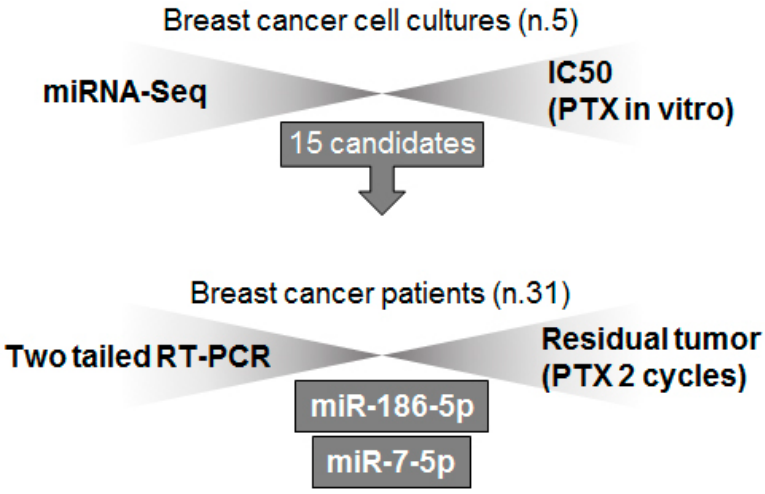

Figure 1. Study design.

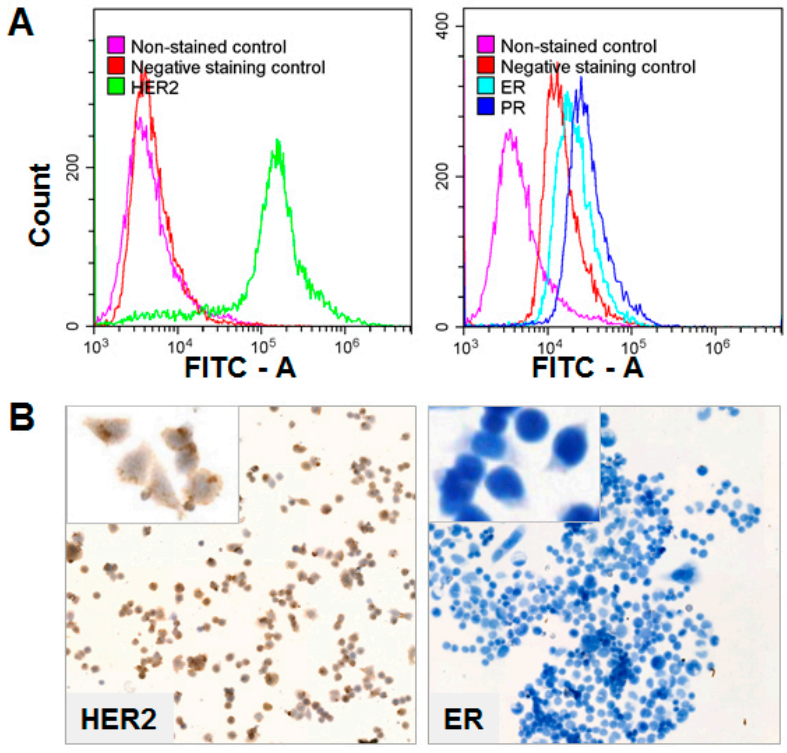

Figure 2. Expression of HER2, ER, and PR in MDAMB453 cell culture evaluated by (A)Flow cytometry; and (B) immunohistochemistry. Images are shown at 10x and 40x magnification.

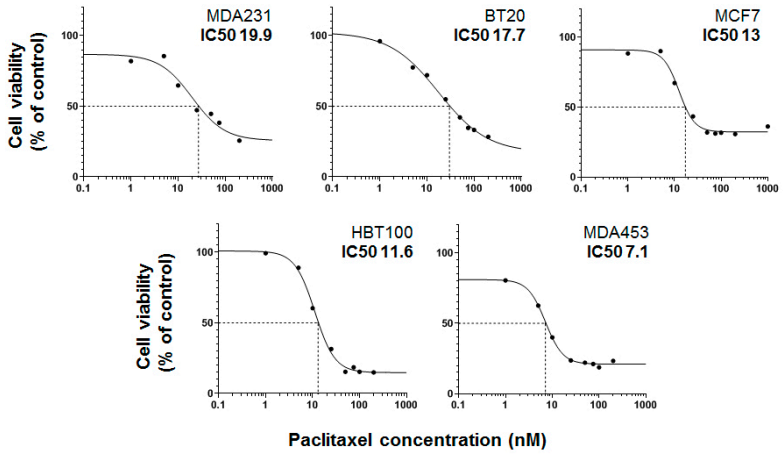

Figure 3. Sensitivity of different BC cell cultures to PTX *in vitro* evaluated by dose-dependent cell viability assay. PTX: Paclitaxel.

The PTX IC50 (IC50-PTX) values in tested cell lines varied considerably, ranging from 7.1 nM to 19.9 nM. MDAMB231 cells (derived from basal-like BC) with specific stellate morphology and low expression levels of ER, PR, and HER2 were relatively resistant to PTX. The BT20 cell line also established from basal-like tumors with similar morphological properties and receptor status was resistant, but slightly lesser (IC50-PTX: 17.7 nM). Maximal sensitivity to PTX was observed in MDAMB453 cells derived from luminal BC with detectable levels of nuclear sex hormones receptors and high expression of the surface receptor, HER2. Both the MCF7 [ER (+)/PR (+)] derived from luminal BC and normal mammary epithelium cell line HBL-100 showed intermediate PTX sensitivity. Thus, cell lines derived from basal-like tumors with low levels of growth factor receptors were relatively resistant to PTX, whereas cell cultures derived from luminal BC or normal mammary epithelium with higher levels of growth factor receptors were more sensitive to PTX.

miRNA expression profiling in BC cell lines

The main goal of our *in vitro* experiments was to evaluate the possible link between miRNA expression patterns and inherent sensitivity to PTX. To assay a broad profile of cellular miRNAs, we performed sequencing. After bioinformatics analysis of miRNA sequencing data, 844 miRNAs were identified at least in one of five cell lines [Supplementary Table 1], with 705 to 750 different miRNAs obtained in each cell line. The total number of reads for each miRNA in each cell type varied from zero to 900,000, indicating a broad range of miRNA expression. Comparative representation of different miRNAs in analyzed samples is graphically shown in Figure 4A using the log scale on X-axis.

Thus, only 250 miRNAs in BT20 cells were quantified by more than 100 reads-292 miRNAs-in HBL100 cell culture, 265 miRNAs in MCF7, 247 miRNAs in MDAMB231, and 245 miRNAs in MBAMD453. Only 145 miRNA molecules reached 100 reads in all five cell lines tested, suggesting that relatively small numbers of miRNAs are represented typically and abundantly in studied cells. These miRNAs play a role in controlling basal cellular functions. Other molecules are less abundant and more variable across cell lines; these miRNAs can mediate temporal expressional alterations and control specific functions.

Next, we estimated the linear correlation between two sets of data (IC50-PTX *vs.* normalized read count for each miRNA) in five cell lines using the Pearson correlation coefficient (Pearson's r). This coefficient exceeded the value of 0.8 for 48 miRNAs and exceeded 0.9 for 18 miRNAs including cases in both direct and inverse relationships [Supplementary Table 1]. An example of such correlation for miR-27a is

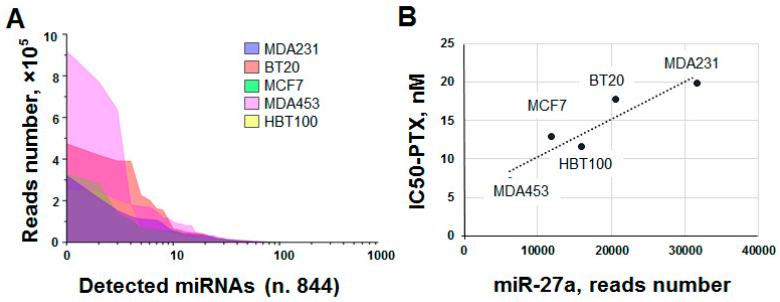

Figure 4. Sequencing of miRNAs in BC cell lines. (A) Overall frequency distribution of detected miRNAs schematically presented using OriginPro 9.1 software; and (B) representative example of the correlation between miRNA-27a expression level and IC50-PTX presented graphically using Microsoft Excel 2016. BC: Breast cancer; PTX: paclitaxel.

illustrated in Figure 4B. We concluded that these molecules might be involved in the response of cells to PTX. In this case, their expression level and functionality might predict the cytostatic effect of the drug. To narrow the list and minimize the number of random matches, relevant scientific data analysis was performed using the PubMed library with the keyword combination of "breast cancer AND paclitaxel AND miR-XXX." Fifteen miRNAs, listed in Table 2, along with the value of Pearson's r and relevant references, were selected for expressional analysis in BC tissue samples.

Expressional analysis of candidate miRNAs in BC tissue samples

BC tissue samples were obtained through diagnostic core biopsy and used for routine histological and IHC evaluation. The patients assigned to the PTX-based NAT group were included in the study and samples of their BC tissue were used for expressional assessment of the selected miRNAs. Characteristics of patients and tumors are presented in Table 3.

Tumor size (before and after two cycles of PTX) was measured by whole breast ultrasound (US) in three dimensions (width, W; length, L, and height, H) and tumor volume was approximated assuming a rounded shape using the following equation: $V = 4/3 \cdot \pi \cdot 1/2W \cdot 1/2L \cdot 1/2H$. Neoadjuvant chemotherapy was started with an intravenous infusion of PTX at a dose of 80 mg/m^2 weekly as recommended by the national standard[48]. The primary effect of therapy was evaluated after two cycles of PTX. Residual tumor volume after two cycles of PTX-based therapy (residual tumor size, RTS) was expressed as a percentage of its initial volume using the following equation: $RTS = (100 \times V2)/V1$. All patients underwent dynamic observation and received optimal therapeutic regimens based on their complex parameters [Table 3]. After NAT was completed, all patients underwent surgery (mastectomy) and the ultimate result of therapy was evaluated histologically [Table 3].

RTS after two cycles of PTX was a parameter reflecting the effect of a single drug. Interestingly, this parameter had an almost normal (Gaussian) distribution among the analyzed group of patients, reflecting the variability of intrinsic sensitivity of BC to PTX [Figure 5A].

Moreover, residual tumor size expressed as a percentage of initial tumor size directly correlated with Ki67 expression [Figure 5B]. Interestingly, we did not find any correlation between the results of histological evaluation of the completed scheme of NAT and Ki67 expression. This observation may indicate the cumulative nature of the action of PTX when an actively proliferating tumor requires long-term exposure to the drug, while a slowly proliferating tumor is suppressed faster. If this explanation is reasonable, it may take more than two weeks for PTX to exhibit a clinical effect.

Table 2. List of miRNAs selected for expressional analysis in BC tissue samples

miRNA	miRNA expression (reads number) in specific cell lines					Pearson's r*	Ref.
	MDA231	BT20	MCF7	HBL100	MDA453		
125b-5p	1391	2045	3818	5615	12236	-0.93	[15,35]
145-5p	69888	22409	4489	9	69	0.83	[35]
16-5p	5797	6116	2798	160	115	0.93	[19]
186-5p	11	74	202	625	1161	-0.93	[36]
191-5p	10191	5735	2128	250	5	0.93	[37]
200c-3p	182	56	528	387	1066	-0.91	[38,39]
221-3p	60509	40509	813	10509	222	0.91	[40]
24-3p	22916	19121	1150	11916	1127	0.87	[41]
27a-3p	31571	20618	11817	15958	6216	0.93	[42]
29-3p	10	11	5	5	1	0.97	[42]
30a-5p	6182	5129	290	2144	65	0.91	[43]
34a-5p	8112	7971	1328	3246	216	0.93	[44]
451a-5p	51371	47166	23124	1321	4982	0.92	[16]
7-5p	135	199	370	586	692	-0.97	[45]
93-5p	6760	18488	18175	28622	80353	-0.87	[46,47]

*Pearson coefficient for linear correlation between two sets of data (IC50-PTX *vs.* normalized read count for each miRNA) in five cell lines. PTX: Paclitaxel.

Expressions of 15 selected microRNA were assayed in all 31 samples of BC tissues obtained by core biopsy before the start of NAT. Results were normalized to average Ct (average of 465 individual values, 31 × 15). Subsequently, we attempted to identify the correlation between the expression of each selected miRNA and the known characteristics of the tumor. Patients were distributed into two groups based on tumor residual value after two cycles of PTX, i.e., more (*n.* 15) and less than 50% (*n.* 16). Expressions of miR-185 and miR-7 were higher in the tumor group more sensitive to PTX [Figure 6].

We did not find any correlation between the expression of other miRNAs tested and the effect of PTX. Finally, we did not find any correlation between candidate miRNA expression and results of post-surgery histological evaluation of NAT's effect using either the Miller&Payn system or Residual Cancer Border classification. When the analysis was applied to 18 patients treated only with PTX in the monotherapy regime, a statistically significant difference in miR-186-5p expression level was observed between groups of patients with different Residual Cancer Border statuses [Figure 7].

DISCUSSION

Several comprehensive genome-wide studies revealed alterations of miRNA profile in BC due to PTX treatment. The list of miRNAs affected by taxane includes molecules implicated in intrinsic resistance[21,22]. MiRNAs apparently involved in acquired taxane resistance were identified by comparative analysis of miRNA profiles of docetaxel-resistant BC cell lines and docetaxel-sensitive parental cells[49]. However, the results of different genome-wide investigations show negligible overlaps, highlighting the necessity of their confirmation by combining *in vivo* and *in vitro* approaches. Other studies combine BC tissue assessment and *in vitro* experiments, which usually focus on the molecular function of certain miRNAs only (12-17) and aim to identify new approaches to overcome taxane resistance.

This study aimed to identify the most reliable prognostic miRNA markers. As an example of a similar approach, the assay of global miRNA expression in BC tissue of patients with and without recurrence after

Table 3. Characteristics of BC patients (*n.* 31) included in the study

Characteristics		Groups	*N* (total number 31)	%
Clinical status	Age	< 50	18	58
		≥ 50	13	42
	Clinical T	T 1-2	25	81
		T 3-4	6	19
	Clinical N	N 0	6	19
		N +	25	81
Histology (trepan biopsy)	Grad	G 1-2	22	71
		G 3-4	9	29
	Ki67	< 20	8	26
		≥ 20	23	74
	ER	Pos	29	94
		Neg	2	6
	PR	Pos	26	84
		Neg	5	16
	HER2	Pos	3	10
		Neg	28	90
Neo-adjuvant therapy*	6-12 PTX		18	58
	6 PTX - AC		4	13
	12 PTX - AC		9	29
PTX effect evaluation (US)	Residual tumor size after 2 cycles of PTX (% from initial volume)	< 50	14	45
		≥ 50	17	55
Completed scheme therapy effect evaluation (histology)	Miller and Payne system	Grade 1-2	8	26
		Grade 3-4	20	65
		Grade 5	3	10
	Residual cancer border class	I	1	3
		II	16	52
		III	11	35
		pCR	3	10

*The schemes of neoadjuvant chemotherapy are shown by number of PTX cycles applied as monotherapy followed by combination of doxorubicin and cyclophosphamide (AC). PTX: Paclitaxel.

endocrine therapy followed by validation of selected miRNAs identified miR-30s, -30b, -182, and -200c as independent predictors of clinical benefit from endocrine therapy[50]. We attempted to identify miRNA predictors of BC cell sensitivity to PTX, and an advantage of our study was a combination of experimental and observational approaches. First, we selected miRNAs whose expression levels were correlated with PTX sensitivity of BC cells *in vitro*. Second, we explored the correlation between the expression of these miRNAs in BC tissues and PTX-based NAT effect. We believe that this strategy helped us to increase confidence in the obtained results and identify miRNAs truly involved in the response of BC cells to PTX. As the mechanism of action of PTX is not associated directly with HR and/or HER2 status[51,52], in our study, we analyzed BC tissue specimens from patients with different subtypes of BC, mostly HR+ /HER2-, and we did not attempt to evaluate any correlation between PTX effect and BC subtypes. The design of our study allowed the evaluation of the link between high levels of miR-186-5p and miR-7-5p expression and the effect of two weekly cycles of PTX. The effect of the complete scheme of NAT was associated with high expression of miR-186-5p in a group of patients treated with PTX only. In patients assigned to the neoadjuvant polychemotherapy (PTX-AC) group, no correlation between tested miRNA expression and

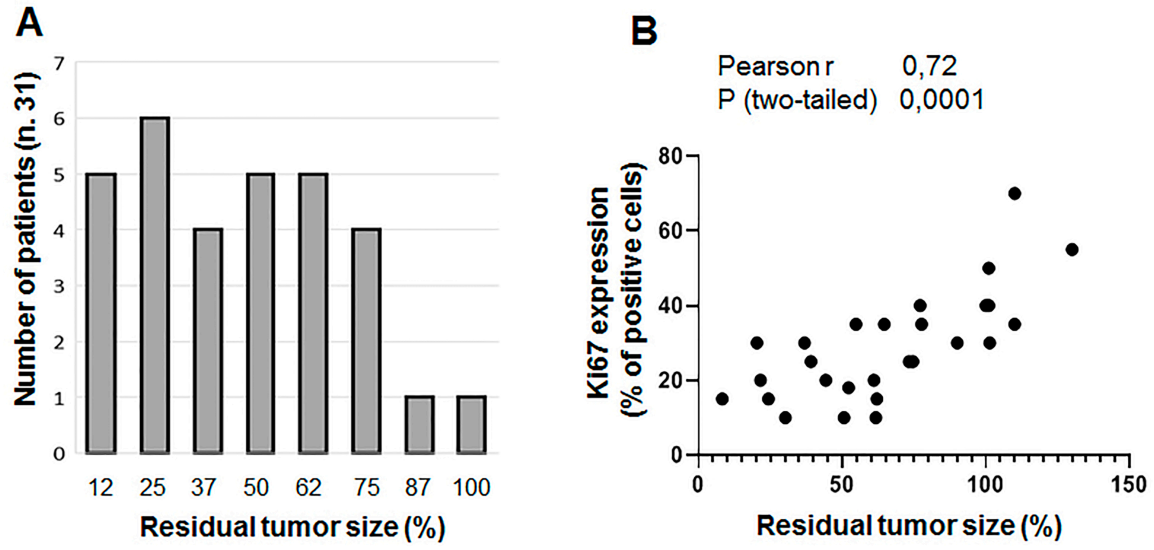

Figure 5. US-based evaluation of PTX-based therapeutic effect. (A) Distribution of patients according to size of residual tumor after two cycles of PTX; and (B) correlation between residual tumor size and Ki-67 expression in specimens from diagnostic biopsy. Both graphs were plotted using GraphPad Prism software. PTX: Paclitaxel.

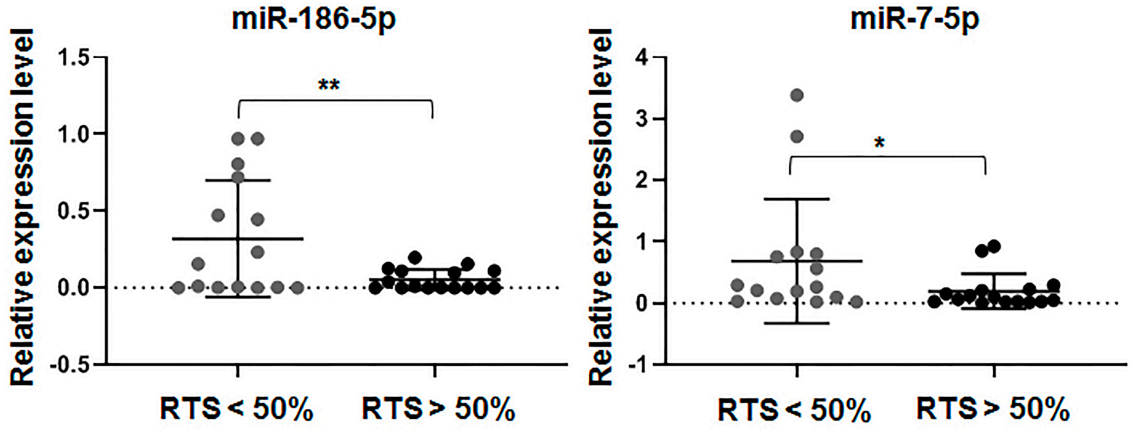

Figure 6. Expression of miRNA (miR-186-5p and miR-7-5p) in BC tissues of patients (*n.* 31) distributed into two groups with the differential effect of PTX-based therapy evaluated as the relative RTS. Statistical significance was estimated by the Mann-Whitney test ($^{*}P < 0.05$; $^{**}P < 0.005$). Graphs were plotted and statistical analysis was performed using GraphPad Prism software. BC: Breast cancer; PTX: paclitaxel; RTS: size of tumor residual.

residual cancer border classes was found. This might highlight the specific association between miR-186-5p expression and PTX sensitivity.

The role of miRNA-186 in cancer is well-studied. Recent reviews have described the dual properties implicated in carcinogenesis and the involvement in various cellular processes[53,54]. In the context of BC, miR-186 was reported as an onco-miR that mediates highly aggressive and metastatic phenotype of tumor[55]. However, miR-186 sensitized non-small lung cancer (*in vitro* and *in vivo*) to PTX by modulating MAPK activity[56]. After computational prediction of the miR-186 binding site in 3'UTR of ABCB1, the

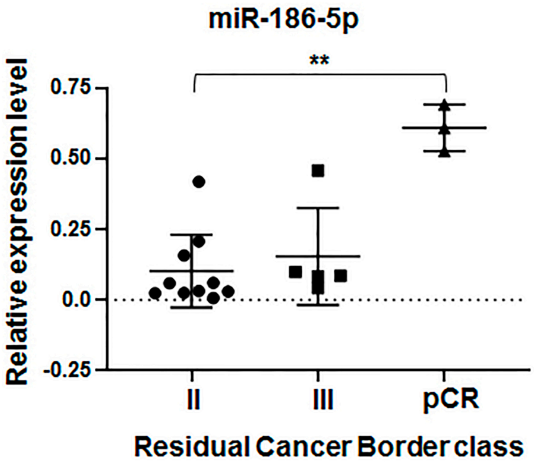

Figure 7. Expression of miR-186-5p in BC tissues of patients (*n.* 18) distributed into three groups with differential effect of complete scheme of PTX-based therapy evaluated after surgery using Residual Cancer Border Classification. Statistical significance was estimated by the Kruskal-Wallis test ($^{**}P$ < 0,005). Graphs were plotted and statistical analysis was performed using GraphPad Prism software. PTX: Paclitaxel.

mechanism of miR-186-mediated sensitivity to PTX was evaluated in *in vitro* models of ovarian cancer[57]. As demonstrated here, the expression of miR-186 was higher in BC cell lines sensitive to PTX and in samples of BC effectively treated by PTX. Therefore, miR-186 may sensitize BC cells to PTX through mechanisms described previously for other cancers.

The link between miR-7 expression and PTX sensitivity observed in our study is consistent with results published by other groups. Interestingly, the sensitization to PTX by miR-7 was reported in the same cancer types as miR-186, including non-small cell lung and ovarian cancer[58,59]. The mechanisms of miR-7 action were non-related to microtubular apparatus and had universal character (regulation of EGFR/ERK signaling). miR-7 was significantly overexpressed (fold change > 3) in response to PTX treatment in a cell line (FaDu) derived from hypopharyngeal tumor[60], reflecting the implication of this molecule in the adaptive reaction of cells to the drug. As reported recently[45], in BC cells, miR-7 reversed resistance to PTX by targeting both the multidrug resistance-associated protein 1 (MRP1) and anti-apoptotic B cell lymphoma 2 (BCL2).

In conclusion, presented results showed that miR-185 and miR-7 expressions are higher in PTX-sensitive BC cells than in resistant cells. It was demonstrated for the first time that the overexpression of these molecules in BC tissues is associated with sensitivity to PTX, whereas their downregulation may reflect resistance to PTX applied as monotherapy. The innovative potential of the presented results lies in the possibility of developing new approaches for predicting the effect of PTX therapy based on the analysis of miRNAs in biopsy material. Since different miRNAs reflect the intrinsic resistance of BC to different drugs, a miRNA prediction panel can be developed and proposed as an additive approach for the personalized choice of NAT regimen.

DECLARATIONS

Authors' contributions

Patients evaluation and selection, collection and analysis of clinical data: Apollonova V

Cell culture experiments including WST test and flow cytometry: Plevako D
RNA sequencing including RNA isolation, small RNA library preparation, sequencing and data analysis: Garanin A
Cell culture experiments including WST test, immunocytochemistry, RNA isolation and RT-PCR: Sidina E
Sequencing data analysis and visualization: Zabegina L
Curation of all RT-PCR experiments including evaluation of specific miRNA Assay Kits, adaptation of protocols and data analysis: Knyazeva M
Histological evaluation of core biopsy material, preparation of slides from paraffin blocks: Smirnova V
Histological evaluation of core biopsy material: Artemyeva A
Study design, clinical evaluation of patients: Krivorotko P
Study design, project administration and manuscript writing: Malek A

Availability of data and materials
Not applicable.

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Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of N.N. Petrov National Medical Research Center of Oncology (protocol No.1 from 2021.01.28). Informed consent was obtained from all subjects involved in the study.

Consent for publication
Not applicable.

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Cancer Drug Resistance

Review

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Tumor-intrinsic metabolic reprogramming and how it drives resistance to anti-PD-1/PD-L1 treatment

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Abstract

The development of immune checkpoint blockade (ICB) therapies has been instrumental in advancing the field of immunotherapy. Despite the prominence of these treatments, many patients exhibit primary or acquired resistance, rendering them ineffective. For example, anti-programmed cell death protein 1 (anti-PD-1)/anti-programmed cell death ligand 1 (anti-PD-L1) treatments are widely utilized across a range of cancer indications, but the response rate is only 10%-30%. As such, it is necessary for researchers to identify targets and develop drugs that can be used in combination with existing ICB therapies to overcome resistance. The intersection of cancer, metabolism, and the immune system has gained considerable traction in recent years as a way to comprehensively study the mechanisms that drive oncogenesis, immune evasion, and immunotherapy resistance. As a result, new research is continuously emerging in support of targeting metabolic pathways as an adjuvant to ICB to boost patient response and overcome resistance. Due to the plethora of studies in recent years highlighting this notion, this review will integrate the relevant articles that demonstrate how tumor-derived alterations in energy, amino acid, and lipid metabolism dysregulate anti-tumor immune responses and drive resistance to anti-PD-1/PD-L1 therapy.

Keywords: Immunotherapy resistance, tumor-immune microenvironment, immune checkpoint blockade, energy metabolism, amino acid metabolism, lipid metabolism

INTRODUCTION

The development of immune checkpoint blockade (ICB) therapies revolutionized cancer treatment across a variety of indications. Immune checkpoints are necessary for the controlled initiation and termination of immune responses as well as for the maintenance of self-tolerance, which are critical in preventing autoimmunity[1]. However, tumors leverage this checkpoint system to inappropriately dampen the immune response and facilitate immune escape[1]. Continuous antigen stimulation drives the upregulation of checkpoint receptors on $CD8^+$ T cells[2], while tumor cells exploit a variety of mechanisms to upregulate checkpoint ligands. Therefore, blocking the interaction between immune checkpoint receptors and ligands reinvigorates $CD8^+$ T cell function to elicit tumor cell killing. There are several ICB therapies that are currently utilized in the clinic, but the most well-studied are anti-programmed cell death protein 1 (anti-PD-1), which is predominantly found on T cells, and anti-programmed cell death ligand 1 (anti-PD-L1), which is expressed on tumor and myeloid cells[3]. While anti-PD-1/PD-L1 treatments are widely used, a substantial number of patients are resistant to this type of therapy[4], prompting researchers to identify resistance mechanisms that drive inadequate outcomes. Response to ICB is largely dependent on the existing profile and infiltration of immune cells within the tumor, specifically $CD8^+$ T cells, because they are the main contributors to anti-tumor effects[4]. Therefore, modulating the tumor-immune microenvironment (TIME) to enhance $CD8^+$ T cell infiltration and function, in combination with current ICB therapies, serves as an attractive approach to increase efficacy and overcome resistance.

The intersection of cancer and metabolism has been at the forefront of oncology research for several decades. Otto Warburg and his identification of the Warburg effect, wherein malignant cells exhibit a metabolic shift from oxidative phosphorylation to glycolysis[5], ignited massive research efforts towards uncovering the metabolic reprogramming that occurs in tumors. These efforts led to the classification of dysregulated tumor cell metabolism as one of the hallmarks of cancer in 2022[6]. Therefore, altered metabolism of lipids, amino acids, carbon, and nucleotides, to name a few, are highly implicated in the development and progression of cancer[7]. More recently, this field of onco-metabolism has expanded to include the immune system, given its role in regulating tumorigenesis. Immune cells and their subtypes have different metabolic requirements during activation, differentiation, and expansion[8], wherein alterations in the extrinsic metabolome at any of these stages can lead to immune cell dysfunction. The TIME is an objectively harsh environment for many cell types due to its acidity, hypoxia, nutrient deprivation, and accumulation of inhibitory metabolites[9]. To the advantage of the tumor, malignant and immunosuppressive cells, such as T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), and macrophages, are better adapted to this oppressive environment compared to anti-tumor $CD8^+$ T cells[10]. These conditions, which are largely facilitated by cancer cells, heavily contribute to decreased $CD8^+$ T cell infiltration and function.

There is mounting evidence that tumor-intrinsic metabolic reprogramming has a profound effect on the recruitment and function of various immune cell types within the TIME. As such, it is necessary to identify ways to specifically target malignant cell metabolism to enhance the efficacy of ICB. The scope of this review article will aim to cover the current literature that demonstrates how tumor-derived alterations in energy, amino acid, and lipid metabolism within the TIME mediate $CD8^+$ T cell dysfunction and how targeting these pathways combats resistance to anti-PD-L1/PD-1 treatment.

ENERGY METABOLISM

Energy metabolism includes a complex network of biochemical pathways that contribute to sustained cellular function through the production of adenosine triphosphate (ATP). Some of these processes include glycolysis, the tricarboxylic acid (TCA) cycle, and fatty acid b-oxidation. A shift in energy metabolism

towards the Warburg effect in malignant cells generates high levels of lactic acid, while consuming and producing ATP/adenosine diphosphate (ADP) and oxidizing and reducing nicotinamide adenine dinucleotide (NAD). While concurrently studying lactate, adenosine, and NAD^+ in the context of energy metabolism is important, each individual metabolite uniquely influences the function of malignant and immune cells within the TIME. Therefore, this section will focus on how the altered metabolism of lactate, adenosine, and NAD^+ by tumor cells impacts the anti-tumor immune response by $CD8^+$ T cells and contributes to anti-PD-1/PD-L1 resistance.

Lactate

Lactate is predominantly formed through glycolysis, wherein lactate dehydrogenase (LDH) reduces pyruvate to lactic acid, which then dissociates into hydrogen (H^+) and lactate ions [Figure 1]. To a lesser extent, glutaminolysis also drives pyruvate formation, resulting in lactic acid production[11]. Lactate and H^+ are exported through proton-linked monocarboxylate transporters 1-4 (MCT1-4)[12], wherein export is highly dependent on the existing concentration of extracellular lactate[13]. Intracellular lactate levels are also modulated by import through MCT1[14]. Extracellular lactate facilitates intracellular signaling by binding to hydroxycarboxylic acid receptor 1 (HCAR1), which regulates a variety of downstream oncogenic pathways, such as cell proliferation, migration, and invasion[15]. Accumulation of H^+ via lactic acid production contributes to the acidity of the TIME, which promotes an immunosuppressive milieu[16]. Conversely, lactate ions have both tumor-promoting and -inhibiting effects in $CD8^+$ T cells.

T cells require adequate levels of lactic acid for proper development and function[17,18], but excess amounts in the TIME and intracellularly promote dysfunction. Tumor-derived lactic acid accumulation within the TIME inhibits T cell proliferation and cytokine production by altering redox homeostasis[19]. Specifically, lactic acid downregulates T cell production of both reactive oxygen species (ROS) and the antioxidant glutathione[19]. While excess amounts of ROS promote oxidative stress, low levels are important for T cell activation and signaling[20], suggesting that tumor-derived lactic acid inhibits T cell functions by ablating ROS formation. Additionally, overabundance of lactic acid in the TIME prevents T cell export of lactate and H^+ ions because of the unfavorable concentration gradient, and subsequent accumulation promotes intracellular acidification and decreases effector function[21]. In particular, intracellular acidification in T cells due to tumor-derived lactic acid production prevents the expression of nuclear factor of activated T cells (NFAT)[22], a family of transcription factors that mediate T cell development[23]. In $CD8^+$ T cells, decreased NFATC1 expression reduces IFNg production, whereas inhibiting lactate dehydrogenase A (LDHA) reduces intracellular acidification and restores $CD8^+$ T cell function and tumor infiltration[22]. Similarly, the hypoxic nature of the TIME drives upregulation of LDHA in $CD8^+$ tumor-infiltrating lymphocytes (TILs), leading to excess intracellular lactic acid, which then inhibits IFNg and granzyme B production[24] and T cell expansion[18]. Upon chronic antigen stimulation, $CD8^+$ T cells will progress through progenitor exhausted and terminally exhausted states, with the latter resulting in dysfunction and the inability to elicit anti-tumor effects[25]. Therefore, there has been a significant focus on promoting the expansion of non-exhausted states and inhibiting the progression into terminal exhaustion to reinvigorate the anti-tumor response. Researchers found that treatment of $CD8^+$ T cells with IL-21 promotes expansion but does not drive T cells towards an exhausted state, like IL-2[18]. Moreover, IL-2, but not IL-21, induced metabolic reprogramming in T cells to favor glycolysis and shunt pyruvate towards lactic acid formation[18]. Treatment with IL-2 and LDH inhibitor invoked a shift from glycolysis towards oxidative phosphorylation, and IL-2 or IL-21 treatment in combination with LDH inhibitor increased stem cell memory T cell formation and reduced tumor growth[18]. These data demonstrate that tumor-derived lactic acid can directly or indirectly inhibit T cell function and anti-tumor immune response.

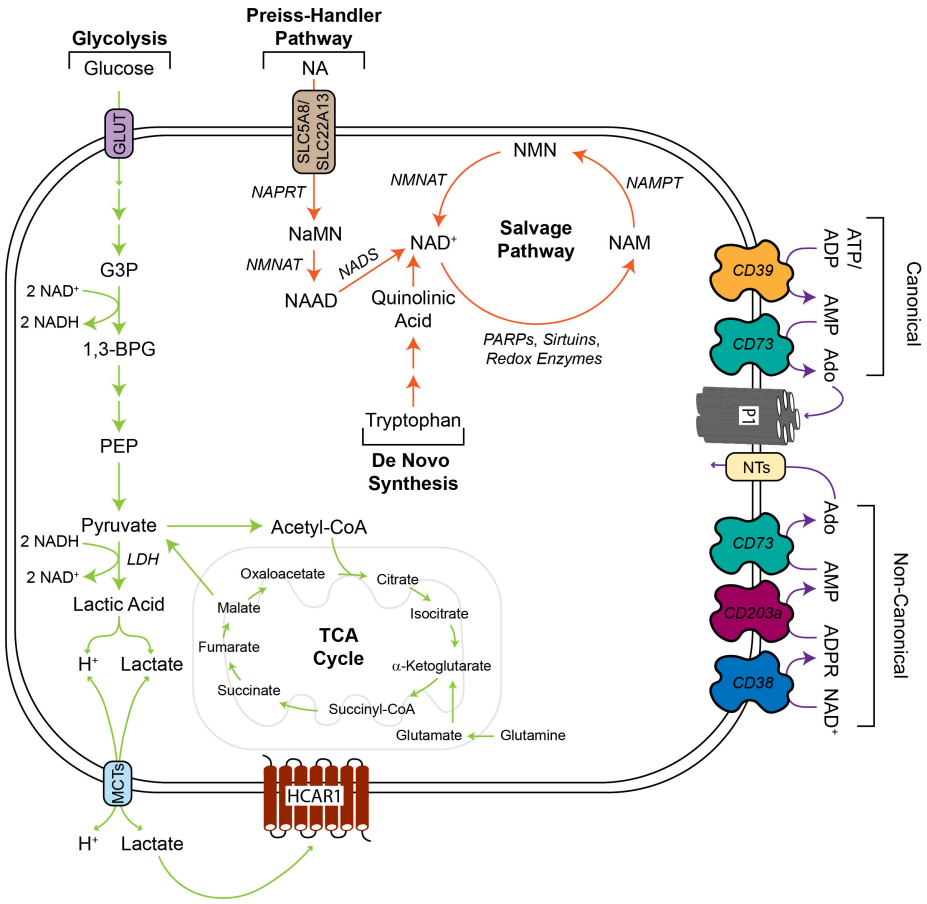

Figure 1. Energy metabolism pathways for lactate, adenosine, and NAD^+. Pyruvate is generated predominantly through glycolysis, but the TCA cycle also contributes to pyruvate production via conversion from malate. LDH catalyzes the reaction to convert pyruvate to lactic acid, which dissociates into H^+ and lactate ions that are exported and imported through MCTs. Alternatively, pyruvate can be converted to acetyl-CoA to participate in the TCA cycle to drive energy metabolism. In the TIME, H^+ contributes to the low pH and lactate facilitates a variety of intracellular signaling pathways by binding to HCAR1. Extracellular adenosine is formed through both the canonical and non-canonical pathways. The canonical pathway utilizes CD39 to convert ATP or ADP to AMP and CD73 to convert AMP to adenosine. The non-canonical pathway metabolizes NAD^+ to ADPR through CD38, ADPR to AMP through CD203a, and finally, AMP to adenosine via CD73. Extracellular adenosine binds to P1 to initiate intracellular signaling pathways or is imported through NTs. Note: adenosine generated by the canonical and non-canonical pathways participates in both P1 signaling and NT import. NAD^+ is formed through the Preiss-Handler pathway, de novo synthesis, salvage pathway, and various enzymatic reactions in energy metabolism, such as PEP to pyruvate. The Preiss-Handler pathway imports NA and forms NAD^+ through a series of enzymatic reactions. Do novo synthesis of NAD^+ results from the metabolism of tryptophan and the salvage pathway recycles NAM to regenerate intracellular NAD^+ levels. NAD^+ serves as a co-factor for many enzymes and participates in redox reactions, such as pyruvate to lactic acid. Ado: Adenosine; ADP: adenosine diphosphate; ADPR: adenosine diphosphate ribose; AMP: adenosine monophosphate; ATP: adenosine triphosphate; GLUT: glucose transporter; G3P: glycerol-3-phosphate; H^+: hydrogen; HCAR1: hydroxycarboxylic acid receptor 1; LDH: lactate dehydrogenase; MCTs: monocarboxylate transporters; NA: nicotinic acid; NAAD: nicotinic acid adenine dinucleotide; NAD^+: nicotinamide adenine dinucleotide; NADS: NAD^+ synthetase; NAM: nicotinamide; NaMN: nicotinic acid mononucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NAPRT: nicotinic acid phosphoribosyltransferase; NMNAT: nicotinamide mononucleotide adenylyltransferase; NMN: nicotinamide mononucleotide; NTs: nucleoside transporters; PARP: poly (ADP-ribose) polymerase; PEP: phosphoenolpyruvate; P1: type 1 purinergic receptors; TCA: tricarboxylic acid; 1,3-BPG: 1,3-Bisphosphoglycerate.

Lactate serves as a carbon source in both tumor and T cells[26-28], but like with any metabolite, overabundance dampens cellular functions. In T cells, increased lactate metabolism depletes NAD^+ levels by reducing it to NADH, preventing the downstream glycolytic processes that rely on NAD^+[29]. Similarly, reduced glycolytic flux in T cells diminishes serine production, which is critical for T cell proliferation[29]. Moreover, tumor-derived lactate promotes depletion of NAD^+ in naïve T cells, resulting in translation inhibition of FIP200, which forms one subunit of the ULK kinase complex that regulates autophagy[30]. FIP200 is selectively lost in naïve T cells from ovarian cancer patients, wherein autophagy is suppressed,

leading to mitochondrial dysfunction and, ultimately, apoptosis[30]. Genetic ablation of FIP200 in naïve T cells reduced $CD8^+$ and $CD4^+$ T cell infiltration and IFNg production[30]. Recently, tumor-derived lactate was also found to diminish TCA-intermediate recycling in $CD8^+$ T cells by inhibiting pyruvate carboxylase, which shunts pyruvate to oxaloacetate[31]. Pyruvate carboxylase is exceedingly important to maintain TCA cycle anaplerosis in $CD8^+$ T cells because succinate is diverted from the TCA cycle to participate in autocrine signaling[31]. In addition to tumor-derived lactate suppressing $CD8^+$ T cell function, it also drives the expansion and function of immunosuppressive cells. Tregs inhibit the function of anti-tumor immune cells and require lactate to maintain their suppressor functions in the harsh TIME[32,33]. Moreover, lactate produced by cervical cancer cells supports immunosuppressive macrophages by regulating anti-inflammatory cytokine production and HIF1a expression[34]. Taken together, these data highlight that tumor-derived lactate not only directly inhibits effector T cell functions, but also indirectly through supporting immunosuppressive cell populations. As such, multiple reports have examined the feasibility of inhibiting tumor-intrinsic lactate metabolism in combination with anti-PD-1/PD-L1 therapy.

Several correlative studies through bioinformatic analyses have demonstrated that targeting lactic acid metabolism might overcome ICB resistance and yield better patient outcomes. High LDH expression has been evaluated as a selection criterion for and predicting response to ICB therapy[35-39]. Similarly, other lactate-related genes have been correlated with the expression of immune checkpoint proteins, $CD8^+$ T cell infiltration, and resistance to ICB in breast cancer[40]. Moreover, decreased glycolytic flux in melanoma patients treated with anti-PD-1 therapy was associated with increased probability of progression-free survival[41].

In addition to bioinformatics studies, numerous reports indicate that inhibiting tumor-intrinsic lactic acid metabolism in combination with anti-PD-1/PD-L1 therapies combats resistance and increases efficacy. MCT4 is regulated at the mRNA level by the demethylase alkB homolog 5 (ALKBH5)[42]. Genetic or pharmacologic inhibition of ALKBH5 reduces intratumoral lactate concentration and the number of Tregs and MDSCs, but has no effect on the number of infiltrating cytotoxic T cells[42]. Furthermore, utilizing a small molecular inhibitor of ALKBH5 significantly improved the efficacy of anti-PD-1 treatment in murine melanoma tumors[42]. Consistent with the findings that lactic acid benefits immunosuppressive cells, researchers found that lactic acid produced by high-glycolytic tumors drove expression of PD-1 on Tregs, but not $CD8^+$ T cells, leading to anti-PD-1 resistance[43]. However, inhibiting either LDHA in tumors or MCT1 in Tregs combined with anti-PD-1 therapy reversed these effects[43]. In addition to inhibiting lactic acid production and/or lactate import, antagonizing intracellular lactate signaling in malignant cells through HCAR1 also promotes anti-tumor effects[44]. Abrogating HCAR1-mediated lactate signaling sensitized tumors to anti-PD-1 and metformin treatment, leading to reduced tumor volume and increased $CD8^+$ T cell infiltration and IFNg production[44].

While a plethora of evidence supports the notion that lactic acid production by tumors and accumulation in T cells drives oncogenesis, a few reports contradict this idea. In mouse melanoma tumors, blocking the export of lactate and H^+ ions through MCT1 and MCT4 reduced the acidification of the TIME[41]. While blocking MCT1 and 4 in T cells decreased lactate secretion and glucose uptake, it surprisingly did not impair IFNg production[41], which contrasts with other findings that accumulation of intracellular lactic acid promotes acidification and dampens effector functions[21,22,24]. The authors found that inhibiting MCT1 and 4 activities in T cells increased glucose flux through the TCA cycle and increased oxygen consumption, thus providing an explanation as to why $CD8^+$ T cell effector functions were preserved[41]. Moreover, pharmacologically inhibiting MCT1 and 4 in combination with anti-PD-1 treatment resulted in increased efficacy and decreased tumor volume[41]. The results from these findings are indeed surprising given the

mounting evidence that accumulation of lactic acid within T cells dampens their function. Researchers have also found that lactate, when studied separately from H^+ in the form of sodium lactate, induces stemness and tumor infiltration, and reduces apoptosis in $CD8^+$ T cells[45]. Moreover, sodium lactate supplementation in three mouse tumor models showed synergistic effects with anti-PD-1 treatment[45]. A plausible explanation for these somewhat contradictory findings is that variations between the TIMEs of different tumor types metabolically reprogram $CD8^+$ TILs in distinct ways, wherein some tumors drive increased sensitivity of $CD8^+$ TILs to lactic acid. Therefore, it is exceedingly important to delineate the metabolic changes in $CD8^+$ TILs from different tumor types to identify the most effective therapy.

Additional research is needed to tease apart the intricate relationship between lactate, lactic acid, tumor cells, $CD8^+$ T cells, and immunosuppressive cells. Inhibiting tumor-derived lactic acid production seems to generally have anti-tumor effects, due to the detrimental effects of high acidity on the anti-tumor immune cells within the TIME. While lactate ions serve as a carbon source and promote $CD8^+$ T cell stemness, they also benefit immunosuppressive cells and excess amounts can dampen T cell effector functions. Collectively, these data demonstrate that tumor-derived alterations in lactic acid metabolism contribute to ICB resistance and modulating these pathways may augment efficacy, prompting the need for continued research efforts in this field.

Adenosine

Adenosine is formed through two major pathways [Figure 1]. In the canonical pathway, ectonucleoside triphosphate diphosphohydrolase-1 (CD39) hydrolyzes ATP or ADP to adenosine monophosphate (AMP)[46], which is subsequently converted to adenosine by ecto-5′-nucleotidase (CD73)[47]. The non-canonical pathway involves the conversion of NAD^+ to adenosine diphosphate ribose (ADPR) through cyclic ADP ribose hydrolase (CD38); ADPR is then metabolized to AMP via ectonucleotide pyrophosphatase/phosphodiesterase 1 (CD203a), and finally to adenosine through CD73[48]. Extracellular adenosine has several fates; it is converted to inosine via adenosine deaminase, converted back to AMP through adenosine kinase, or binds to type 1 purinergic receptors, which include A1, A2A, A2B, and A3. Both A2A and A2B receptors (A2AR and A2BR) are important for mediating adenosine signaling in immune cells within the TIME[49]. High affinity A2AR is more broadly expressed on immune cells, while low affinity A2BR facilitates the expansion of MDSC populations[50].

Within the TIME, adenosine formation is predominantly mediated by malignant and immunosuppressive cells[51] and the impact of this metabolite on immunosuppression and cancer progression was recently comprehensively reviewed[52]. Under physiological conditions, extracellular ATP and adenosine levels are low[53]. However, during cellular stress, such as hypoxia and nutrient deprivation, intracellular ATP is released and serves as a strong pro-inflammatory mediator by recruiting immune cells[53,54]. On the other hand, adenosine is a potent immunosuppressive metabolite[50]. As such, it is not surprising that tumor cells highly upregulate CD73 and immunosuppressive cells, such as cancer-associated fibroblasts (CAFs), Tregs, and MDSCs, highly upregulate CD39 to facilitate adenosine accumulation within the TIME[52,55-59]. Further, terminally exhausted $CD8^+$ T cells exhibit increased CD39 expression, therefore contributing to the elevated adenosine levels within the TIME[60], and adenosine drives the expansion of Treg populations[61].

Tumor-derived adenosine inhibits $CD8^+$ T cell functions in a myriad of ways. Adenosine triggers IL-10 secretion from cervical cancer cells, leading to downregulation of MHC-I expression and subsequent immune evasion from $CD8^+$ T cells[62]. Increased adenosine production also favors tumor growth, as indicated by the negative correlation between CD73 expression and survival in pancreatic adenocarcinoma human cohorts[63]. Moreover, loss of CD73 in pancreatic ductal adenocarcinoma cell lines leads to increased

activation and IFNg production in $CD8^+$ T cells[63], highlighting the inverse relationship between adenosine and $CD8^+$ T cell function. Adenosine production within the TIME is also regulated by cancer exosomes, which are endosomal-derived extracellular vesicles[64,65]. Specifically, cancer exosomes were found to express CD39 and CD73, leading to inhibition of T cell activation and proliferation in human neuroblastoma samples[66] and bladder, colorectal, prostate, and breast cancer cell lines[67]. Accumulation of adenosine within the TIME also severely hinders tumor infiltration by $CD8^+$ T cells due to adenosine-mediated dysfunction of KCa3.1 channels[68,69]. KCa3.1 is a potassium channel that regulates Ca^{2+} influx, which affects T cell gene expression, activation, and differentiation[70]. Inhibition of KCa3.1 by adenosine reduced T cell migration and cytokine production[69], and decreased KCa3.1 channel activity, but not protein expression, resulting in decreased tumor infiltration[68]. Building on this, the same group later found that anti-PD-1 therapy increased the activity of ion channels KCa3.1 and Kv1.3, leading to enhanced $CD8^+$ T cell infiltration in head and neck squamous cell carcinoma (HNSCC) patient samples[71]. While not the focus of this section, it is important to mention that Treg-derived adenosine also drives $CD8^+$ T cell dysfunction[56,57,72,73]. On the other hand, increased IL-7 signaling in $CD8^+$ T cells inhibits FoXO1 activation, which is a transcription factor that controls T cell proliferation, to overcome the suppressive effects of the adenosine-rich TIME and promote tumor infiltration and expansion[74]. Leveraging these mechanisms might be a viable therapeutic strategy to be used in conjunction with current ICB therapies to overcome resistance.

Adenosine within the TIME engages with the A2A receptor (A2AR) on $CD8^+$ T cells to drive adenosinergic signaling that results in impaired anti-tumor effects[75]. Early studies found that A2AR signaling inhibited T cell activation and proliferation[76], and in the context of cancer, many studies have shown that A2AR signaling promotes immune evasion and T cell dysfunction. In mouse melanoma and fibrosarcoma models, pharmacological inhibition or genetic deficiency of A2AR increases $CD8^+$ T cell tumor infiltration and IFNg production, and reduces tumor growth[77,78]. Moreover, targeted knockdown or antagonizing A2AR increases $CD8^+$ T cell infiltration[79] and decreases Treg infiltration and tumor volume in mouse models of HNSCC[80]. Similarly, administering A2AR agonists during T cell activation impaired cytotoxic function, although proliferative capacity was maintained, and these effects persisted after A2AR agonists were removed[81]. These data demonstrate that even if $CD8^+$ T cells infiltrate the adenosine-rich TIME, adenosinergic signaling reduces their effector functions and renders them incapable of eliminating tumor cells. However, one study showed that complete abrogation of the A2AR gene in $CD8^+$ T cells inhibited expansion and effector functions[75]. In this way, it is important to preserve some degree of A2AR signaling in $CD8^+$ T cells to maintain proper cell function, highlighting that complete deletion of immunosuppressive targets might not produce the most efficacious results.

The studies thus far have demonstrated that tumor-intrinsic adenosine metabolism adversely affects $CD8^+$ T cell function; therefore, it is not surprising that these metabolic alterations also contribute to anti-PD-1/PD-L1 resistance. To date, there are many drugs in the pre-clinical and clinical stages that target CD39, CD73, and A2AR, either alone or in combination with anti-PD-1/PD-L1 therapies[82]. Because it is not feasible to cover all these data, we have chosen to focus on the relevant articles from 2020 until now to demonstrate that modulating adenosine metabolism helps overcome resistance to ICB therapies. Using bioinformatics approaches, researchers showed that adenosine signaling gene signatures are inversely correlated with survival and efficacy of anti-PD-1 treatment across multiple cancer indications[83]. The first-in-human study using an A2AR antagonist with anti-PD-L1 treatment improved the probability of progression-free survival and overall survival, and monotherapy or combination with anti-PD-L1 increased $CD8^+$ T cell infiltration[84]. However, current A2AR antagonists do not perform well in the adenosine-rich TIME, so multiple groups have developed novel A2AR antagonists to increase effectiveness[85,86]. Both

compounds have shown limited toxicity in Phase I clinical trials[85,86], with iTeos Therapeutics' compound demonstrating initial signs of clinical benefit[86]. Dizal Pharmaceuticals' compound was also evaluated in murine models of prostate cancer, where treatment with the novel antagonist and anti-PD-1 significantly reduced tumor volume compared to monotherapy[85].

There are several pre-clinical and clinical studies that demonstrate promising results for targeting CD39 or CD73 in combination with anti-PD-1 or PD-L1. Cancer exosomes expressing CD39 and CD73 drive adenosine accumulation and were also found to promote CD39 expression on macrophages[87]. Macrophage-derived CD39 cooperates with tumor-derived CD73 to increase adenosine levels in the TIME, which drives anti-PD-1 resistance[87]. Targeting CD39 on macrophages in combination with anti-PD-1 therapy abrogated therapeutic resistance and synergistically reduced the volume of murine hepatocellular carcinoma tumors and increased $CD8^+$ T cell infiltration and granzyme B production[87]. Moreover, a first-in-human Phase I clinical trial was conducted in 2020 to assess the efficacy of an anti-CD39 antibody (IPH5201) in combination with anti-PD-L1 treatment[88], and the first patient for the Phase II study was dosed in June 2023[89]. A poster presentation at the European Society for Medical Oncology Immuno-Oncology Summit in 2022 showed pre-clinical data for IPH5201, wherein treatment alone reduced adenosine levels in the TIME of mouse fibrosarcoma tumors[90]. The data also demonstrated that combining anti-CD39, the chemotherapeutic agent gemcitabine, and anti-PD-L1 controlled tumor growth and increased survival better than monotherapy or anti-PD-L1 with gemcitabine in murine colorectal carcinoma tumors[90]. In a clinical study of 44 patients, researchers found no major toxicities when combining an anti-CD39 monoclonal antibody with anti-PD-1 and the chemotherapy regimen FOLFOX for the treatment of gastric cancer or gastroesophageal junction adenocarcinoma[91]. These data are critical first steps in the approval and use of anti-CD39 therapies in combination with anti-PD-1/PD-L1 treatment. The results from a first-in-human Phase I clinical trial with anti-CD73 and anti-PD-L1 recently reported tolerable safety and moderate efficacy[92]. Further, targeting CD73 has also recently been shown to be a promising therapeutic strategy, wherein Phase II clinical trials combining anti-CD73 with anti-PD-L1 elicit increased response rate and progression-free survival compared to anti-PD-L1 monotherapy in patients with non-small cell lung cancer[93]. One thing to consider when targeting CD39 or CD73 is that anti-CD39 treatments not only inhibit adenosine production, but also promote accumulation of immunostimulatory ATP.

In addition to more conventional treatment methods, several unique approaches for inhibiting adenosine metabolism and PD-1 have recently been discovered. Because of the ubiquitous expression of A2AR on T cells, localizing inhibition of A2AR signaling to tumor-infiltrating $CD8^+$ T cells would likely mitigate off-target effects. In this approach, researchers increased tumor oxygenation to relieve the hypoxic conditions that promote tumor-derived adenosine production[94]. Using a photo-modulated nanoreactor, hydrogen peroxide is converted to oxygen within the TIME, leading to decreased adenosine production and abrogated A2AR signaling in $CD8^+$ T cells[94]. Moreover, combination with anti-PD-1 therapy synergistically reduced tumor growth and increased $CD8^+$ T cell infiltration in triple-negative murine breast cancer tumors[94]. In another tumor-targeting approach, researchers utilized cancer-derived exosomes packaged with both a CD39 antagonist and AMPK agonist to inhibit adenosine and promote ATP production, respectively[95]. This method increased $CD8^+$ T cell infiltration and production of granzyme B and IFNg, reduced intratumoral adenosine and Treg populations, and synergized with anti-PD-1 treatment in mouse melanoma models[95]. The final targeted approach used ROS-producing nanoparticles to deliver a CD39 inhibitor[96]. Inducing ROS accumulation in the TIME seems counterintuitive, but like hypoxia, ROS trigger the release of ATP. Therefore, ROS would increase ATP concentration and inhibiting CD39 would prevent adenosine formation, thus remodeling the TIME away from an immunosuppressive state[96]. This method

alone decreased tumor volume and increased $CD8^+$ T cell production of IFNg and, together with anti-PD-1, elicited a more robust anti-tumor effect in murine mammary carcinoma tumors[96].

Collectively, these data strongly demonstrate that tumor-derived adenosine has detrimental effects on $CD8^+$ T cell infiltration and effector functions, thereby contributing to anti-PD-1/PD-L1 resistance mechanisms. As such, there is a compelling need for the continued development of adenosine-targeting drugs that can synergize with current anti-PD-1/PD-L1 therapies to prevent resistance and evoke better patient response.

NAD^+

NAD^+ is comprised of adenosine monophosphate linked to nicotinamide mononucleotide. NAD^+ can be reduced to form NADH or phosphorylated and subsequently reduced to form $NADP^+$ or NADPH, respectively. NAD^+ is synthesized through three pathways: de novo biosynthesis, Preiss-Handler pathway, or the salvage pathway, the latter of which is the predominant way that cells restore NAD^+ levels[97] [Figure 1]. NAD^+ is a co-factor that is involved in a variety of redox and non-redox reactions. In energy metabolism, NAD^+ and its derivatives are indispensable for cellular function because they accept and donate electrons in a variety of metabolic pathways, such as glycolysis, pentose phosphate pathway, TCA cycle, and fatty acid b-oxidation[98]. NAD^+ also acts as a substrate for multiple enzyme families, including sirtuins, PARPs, and ADP-ribosyl cyclases[97]. Moreover, the metabolic pathways of adenosine and NAD^+ are tightly linked through CD38, an ectoenzyme present on the surface of tumor and immune cells, which depletes NAD^+ levels, which ultimately results in adenosine formation[99].

High NAD^+ levels are required in malignant cells to meet their increased energetic demands for rapid growth and proliferation. Therefore, malignant cells will upregulate NAD^+ biosynthesis to replenish intracellular stores, leading to depletion of this metabolite within the TIME. Several enzymes involved in anabolic NAD^+ pathways, such as nicotinamide phosphoribosyltransferase (NAMPT), have been heavily implicated in cancer progression and severity[100]. Moreover, drugs targeting these enzymes have shown promising results in pre-clinical and clinical studies[101]. Targeting tumor-intrinsic NAD^+ metabolism is a promising therapeutic approach because it would restore NAD^+ levels in the TIME, thus allowing T cells to utilize this metabolite to maintain proper function.

NAD^+ is highly important for anti-tumor immune functions and NAMPT is an important regulator of NAD^+ availability. As previously mentioned, NAD^+ and adenosine metabolism are highly linked due to the ability of NAD^+ to be converted to adenosine. Inhibiting NAMPT in tumor cells reduces levels of intracellular NAD^+ and extracellular adenosine, thereby enhancing $CD8^+$ T cell functions[102]. Further, NAMPT expression in $CD8^+$ T cells is necessary to produce NAD^+ and induce anti-tumor effects[103]. In tumor-infiltrating lymphocytes (TILs), NAMPT and NAD^+ levels are lower compared to peripheral T cells[103], suggesting that the TIME induces NAD^+ depletion in TILs, leading to impaired function. Mechanistically, NAD^+ deficiency in TILs drives mitochondrial dysfunction and reduces ATP production, whereas supplementation with nicotinamide (NAM), the substrate of NAMPT, reverses these effects to promote a strong anti-tumor immune response *in vivo*[103]. Interestingly, TCR stimulation in $CD8^+$ T cells leads to a 16-fold upregulation of NAMPT, compared to 1.3-fold upregulation in Tregs[104]. This suggests that $CD8^+$ T cells rely more heavily on NAMPT expression and NAD^+ levels compared to Tregs, giving these immunosuppressive cells an advantage in the NAD^+-depleted TIME. Consistently, Tregs are particularly sensitive to NAD^+-induced cell death[105], and systemic NAD^+ treatment preferentially depleted Tregs, leading to decreased tumor volume[106]. To date, there are several pre-clinical and clinical studies investigating the use of NAMPT inhibitors in both solid and hematologic malignancies[107]. However, systemic inhibition of NAMPT might have profound adverse effects on $CD8^+$ T cell function, decreasing the drugs' efficacy. Perhaps these types of drugs are more effective in cancers that do not have high T cell infiltration but overexpress NAMPT.

In immune cells, CD38 is inversely correlated with NAD^+ levels because it degrades NAD^+ to NAM and ADP-ribose[108,109]. These derivates of NAD^+ are important secondary messengers that regulate intracellular calcium levels and storage, which in turn mediates T cell differentiation and activation[109]. CD38 expression is a marker of T cell exhaustion that contributes to adverse epigenetic modifications in $CD8^+$ TILs[110]. Further, high expression of CD38, PD-1, and CD101 correlates with the inability of $CD8^+$ T cells to undergo epigenetic reprogramming to reverse the exhausted state[110]. Conversely, inhibiting CD38 expression in Tregs and B-regulatory cells induced cell death, but drove proliferation of cytotoxic T cells, likely due to depletion of the immunosuppressive populations[111]. Consistently, mice deficient in CD38 expression exhibited lower Treg numbers as a result of increased NAD^+ levels[106]. CD38 expression on tumor cells has also been implicated in a variety of solid and hematologic malignancies[112-116]. Increased CD38 expression on malignant cells results in acquired resistance to anti-PD-1/PD-L1 therapy by driving $CD8^+$ T cells towards an exhausted state[114]. Moreover, $CD8^+$ T cell function was found to be inhibited by CD38-mediated adenosine production, and anti-PD-L1 and CD38 combination therapy synergistically inhibited the growth of murine lung adenocarcinoma tumors[114]. Currently, there are two approved anti-CD38 monoclonal antibody treatments (Daratumumab and Isatuximab) and one in clinical trials (MOR202) to treat multiple myeloma; however, these drugs do not inhibit the ectoenzymatic activity of CD38, rather they induce antibody-dependent cell-mediated cytotoxicity[117-119]. There are several drugs in pre-clinical stages that target the ectoenzymatic activity of CD38 to increase NAD^+ levels for different diseases[120-122]. While these drugs are not yet being evaluated in the oncologic space, it would be advantageous because inhibiting CD38 is both beneficial for T cells and detrimental for malignant and immunosuppressive cells, thus eliminating the need for cell-specific drugs.

Taken together, these data demonstrate an important role for lactate, adenosine, and NAD^+ in regulating immune cell function and ultimately controlling cancer development and progression. Further, pre-clinical studies show promising results that combining these treatments with existing ICB therapies can remodel the TIME to boost the anti-tumor immune response. Thus, continued pre-clinical and clinical efforts are needed to determine whether resistance to anti-PD-1/PD-L1 therapy is ablated when combined with approved anti-CD39/CD73/A2AR/CD38 treatments.

AMINO ACID METABOLISM

Amino acid metabolism is widely implicated in oncogenesis due to the necessity of amino acids in protein synthesis, epigenetic modifications, and fueling energetic processes. Of the 20 amino acids, only a handful are well-studied in the context of immuno-oncology metabolism and resistance to ICB. Because tryptophan is thoroughly researched in this space and was recently comprehensively reviewed[123], we wanted to focus on amino acids that are sometimes overlooked but still immensely important in regulating cancer development and progression. As such, this section will discuss how tumor-derived alterations in arginine, glutamine, and methionine metabolism contribute to anti-tumor immunity and how modifying the metabolism of these amino acids helps diminish resistance to anti-PD-1/PD-L1 therapy.

Arginine

Arginine is considered a non-essential amino acid in normal cells because it can be imported or synthesized through citrulline metabolism in the urea cycle[124] [Figure 2]. Conversely, arginine is also catabolized through the urea cycle to form urea and ornithine through arginase (ARG) enzymes[124]. Extracellular arginine also participates in the activation of intracellular signaling pathways by binding to G protein-coupled receptor family C group 6 member A (GPRC6A)[125]. While arginine itself is important for many

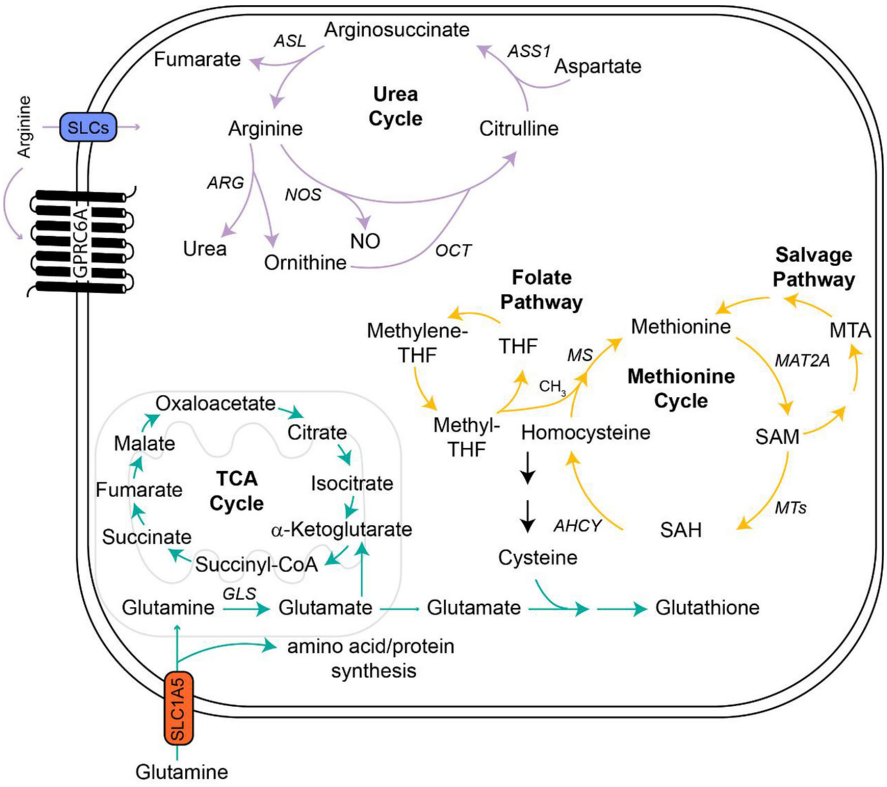

Figure 2. Metabolic pathways of arginine, glutamine, and methionine. Extracellular arginine binds to GPRC6A to drive intracellular arginine signaling or it is imported through various SLC transporters depending on the cell type. Arginine can also be formed through metabolism of citrulline in the urea cycle. Once inside the cell, arginine is catabolized through NOS to form NO or ARG into urea and ornithine, the latter of which is converted back into citrulline to fuel the urea cycle. Glutamine is similarly imported through a variety of SLCs, with SLC1A5 being the predominant transporter on T cells. Intracellular glutamine is used for amino acid/protein synthesis or transported to the mitochondria and converted to glutamate via GLS. In the mitochondria, glutamate is converted to a-Ketoglutarate to fuel the TCA cycle. In the cytosol, glutamate combines with cysteine to form glutathione to combat oxidative stress. Cysteine is generated in part through metabolism of homocysteine in the methionine cycle, which generates methionine for various cellular processes. Methionine is generated by re-methylation of homocysteine through donation of CH_3 by methyl- THF in the folate cycle. Methionine is then converted to SAM, an indispensable methyl donor, and subsequently SAH following loss of the methyl group. SAM is also involved in the methionine salvage pathway that restores intracellular methionine levels. AHCY: Adenosylhomocysteinase; ARG: arginase; ASL: argininosuccinate lyase; ASS1: argininosuccinate synthase 1; CH_3: a methyl group; GLS: glutaminase; GPRC6A: G protein-coupled receptor family C group 6 member A; MAT2A: methionine adenosyltransferase 2A; MS: methionine synthase; MTA: 5′-methylthioadenosine; MTs: methyltransferases; NO: nitric oxide; NOS: nitric oxide synthase; OCT: ornithine transcarbamoylase; SAH: S-adenosyl-L-homocysteine; SAM: S-adenosylmethionine; SLC: solute carrier; TCA: tricarboxylic acid; THF: tetrahydrofolate.

cellular processes, it is also a precursor for the synthesis of polyamines, which are organic compounds that facilitate cell proliferation and are upregulated in a variety of cancers[126-128]. Similarly, nitric oxide synthase (NOS) metabolizes arginine to nitric oxide (NO), which promotes angiogenesis and metastasis, and dampens the immune response[129].

In malignant cells, arginine helps sustain tumor-promoting functions, and arginine starvation results in detrimental effects, such as ROS formation, mitochondrial dysfunction, and cell death[130-135]. Despite this, metabolic rewiring of the urea cycle in tumor cells results in increased ornithine and proline synthesis and decreased arginine synthesis[131]. Moreover, many cancer types have decreased expression of arginosuccinate synthase 1 (ASS1), which catalyzes the penultimate step in arginine synthesis[130]. As such, arginine is considered an essential amino acid in malignant cells, and they must rely on exogenous uptake to sustain their metabolic demands[130-132]. On the other hand, T cells are completely reliant on exogenous arginine

because they do not express ASS1[136,137], meaning they must compete with tumor cells and immunosuppressive cells for arginine.

T cell function is highly disrupted by arginine depletion within the TIME, which is mediated by both malignant cells[138-141] and immunosuppressive cells[142-147]. In T cells, arginine is important in regulating CD3z expression, which is necessary for proper antigen recognition by the TCR-CD3 complex[148-151]. For example, ARG2-dependent depletion of arginine by murine renal cell carcinoma cells leads to decreased expression of CD3z in T cells[139]. Sufficient arginine levels are also necessary during T cell activation because arginine is quickly metabolized to fuel downstream processes[152]. Moreover, decreased systemic arginine levels in Lewis lung carcinoma[150] and arginine depletion via ARG1 from cancer-derived exosomes in ovarian carcinoma[153] inhibit antigen-specific proliferation of $CD8^+$ TILs. Arginine depletion also impairs the effector function of $CD8^+$ T cells by preventing the secretion of IFNg and granzyme B[154,155]. On the other hand, arginine supplementation in $CD8^+$ T cells induces metabolic rewiring from glycolysis towards oxidative phosphorylation to promote proliferation, survival, and anti-tumor responses[152].

Several promising pre-clinical studies have demonstrated that targeting arginine metabolism in combination with anti-PD-1/PD-L1 treatment increases efficacy in overcoming resistance. Employing anti-PD-1 treatment in combination with vaccine inhibition of ARG1 synergistically impaired tumor growth and led to increased $CD8^+$ T cell infiltration in mouse models of colorectal carcinoma and fibrosarcoma[156]. Further, systemic arginine supplementation with anti-PD-1 or PD-L1 treatment increased $CD8^+$ T cell infiltration and exhibited more efficacious results than monotherapy in mouse models of colon carcinoma[157] and osteosarcoma[158]. Utilizing a unique approach, researchers engineered an *E. coli* strain that localizes to the TIME and converts ammonia to arginine[159]. This innovative method promoted continuous arginine supplementation in murine colorectal carcinoma tumors, leading to increased $CD8^+$ T cell infiltration and synergistic anti-tumor effects when combined with anti-PD-L1 treatment[159]. Extensive pre-clinical studies for a novel ARG1/2 inhibitor (OATD-02) have shown promising results alone and in combination with both anti-PD-1 and -PD-L1, and researchers are hopeful this drug will enter first-in-human clinical trials soon[150,160-162]. Moreover, the ARG1 inhibitor CB-1158 entered first-in-human clinical trials in 2017 and was evaluated with anti-PD-1 treatment[163-165]. The results indicate that CB-1158 monotherapy and combination with anti-PD-1 are well-tolerated and elicit a response in solid tumors[163-165].

A considerable amount of evidence demonstrates that tumor-mediated depletion of arginine negatively impacts $CD8^+$ T cell function and the anti-tumor response. Additionally, the enhanced anti-tumor effects seen by combining anti-PD-1/PD-L1 with ARG inhibitors or arginine supplementation demonstrate that altering tumor metabolism could have profound effects on the efficacy of ICB. However, continued pre-clinical and clinical efforts are necessary to identify additional ways to target tumor-derived arginine metabolism and reinvigorate the anti-tumor immune response to improve ICB.

Glutamine

Glutamine has many essential functions, such as supporting the formation of nucleotides and non-essential amino acids, protein synthesis, energy metabolism, and maintaining intracellular redox states[166]. Import of glutamine is facilitated by many transporters, predominantly SLC1A5[136,167] [Figure 2]. Once inside the cell, glutamine is transported to the mitochondria to be converted to glutamate via glutaminase enzymes[166]. In the cytosol, glutamate serves as a precursor for glutathione synthesis, which is a strong antioxidant[166]. The metabolism of glutamine also drives the formation of NADPH, which is critical for restoring the intracellular redox balance by reducing oxidized glutathione[168]. In the mitochondria, glutamate is converted to a-Ketoglutarate to drive the TCA cycle[166].

Many cancers exhibit a dependence on or addiction to glutamine. As such, increased glutaminolysis is highly important for ATP production, redox homeostasis, and activation of various oncogenic signaling pathways in tumor cells[168-170]. Glutamine fuels KRAS signaling in pancreatic adenocarcinoma[168], mTORC1 signaling in osteosarcoma and cervical cancer cells[170], and promotes lipid biogenesis under hypoxic conditions to provide additional energy sources[171]. Hypoxia also drives the mitochondrial import of glutamine to support ATP and glutathione production to combat oxidative stress and promote uncontrolled cell growth[172]. Interestingly, data suggest that some cancers will adapt to the glutamine-deprived TIME and will cease to rely on glutamine. In patient-derived melanoma tumors, for example, excess dietary glutamine inhibits cell growth[173].

T cells require glutamine for a variety of functions during differentiation and development[174]; thus, there is stiff competition between tumor cells and T cells for glutamine consumption. Ligation of CD3 and CD28 on T cells induces glutamine uptake via ERK and calcineurin pathways to sustain T cell activation, proliferation, and cytokine production[175,176]. Interestingly, glutamine is also required for glucose uptake and glycolysis in activated $CD8^+$ T cells, and proper effector functions were dependent on both glucose and glutamine[177]. As such, increasing glutamine availability for T cells, while depriving tumor cells and immunosuppressive cells, has strong anti-tumor effects. For example, selectively inhibiting glutamine uptake in triple-negative breast cancer cells increased $CD8^+$ T cell activation and effector function by promoting glutathione production[178]. On the other hand, non-specific intracellular depletion of glutamine leads to impaired mitochondrial function and $CD8^+$ T cell apoptosis[179], likely due to increased oxidative damage from reduced glutathione production. Data also suggest the temporal importance of glutamine availability in driving T cell function. During TCR stimulation, glutamine deprivation decreases PD-1 and increases Ki67 expression[180], suggesting that glutamine abundance needs to be tightly regulated at various stages of T cell development to ensure proper functionality. As discussed in previous sections, immunosuppressive cells largely thrive in the nutrient-deprived TIME. Specifically, tumor-associated macrophages respond to low glutamine levels by secreting IL-23 to promote Treg proliferation and activation, resulting in diminished $CD8^+$ T cell function[181].

Several reports have demonstrated that inhibiting tumor-associated glutamine metabolism in combination with anti-PD-1/PD-L1 therapies may be a promising approach to restore $CD8^+$ T cell function and overcome resistance. Because glutamine deprivation promotes T cell dysfunction, specifically inhibiting glutamine metabolism in tumor cells would yield the most efficacious results. Two separate groups found that glutamine deprivation in cell lines of human clear cell renal carcinoma[182], human non-small cell lung carcinoma[183], and mouse colorectal carcinoma[183] induced PD-L1 expression, which would theoretically boost anti-PD-L1 response. Byun *et al.* found that anti-PD-L1 monotherapy had almost no effect on tumor volume in murine colorectal carcinoma models[183]. However, tumor-specific inhibition of glutamine uptake and glutaminase activity in combination with anti-PD-L1 therapy strongly induced $CD8^+$ T cell proliferation and granzyme B production, while abating tumor growth[183]. Similarly, another group targeted tumor-derived glutamine enzymes by creating a prodrug that is only activated by TIME-restricted enzymes to limit the cytotoxic effects of systemic glutamine antagonism[184]. This treatment method decreased glycolysis in malignant cells, decreased hypoxia, acidosis, and nutrient depletion within the TIME, and increased activation of and oxidative phosphorylation in $CD8^+$ T cells[184]. In combination with anti-PD-1 therapy, tumor-specific glutamine antagonism synergistically reduced tumor growth and increased survival in murine colorectal carcinoma tumors[184]. Conversely, employing a non-tumor cell specific glutaminase inhibitor does not yield the same efficacious results. Serine/threonine kinase 11 (STK11) phosphorylates AMPK to regulate a variety of downstream pathways, such as cell growth and proliferation, lipid metabolism, and PD-L1 expression[185]. Several studies have shown that STK11 mutations, resulting in loss

of function, are associated with resistance to anti-PD-1 treatment[186-188]. Building on this, one group found that STK11-mutated lung adenocarcinomas from both patient samples and cancer cell lines exhibited increased glutamate production, so they hypothesized that targeting glutaminase would be a viable way to overcome resistance to anti-PD-1 treatment[189]. However, they found that using a non-tumor cell-specific glutaminase inhibitor in combination with anti-PD-1 severely impeded $CD8^+$ T cell clonal expansion and anti-tumor functions, and anti-PD-1 efficacy was dependent on intact $CD8^+$ T cell glutaminase activity[189].

These data demonstrate a promising future for targeting glutamine metabolism to bolster $CD8^+$ T cell effector function and combat ICB resistance. However, it also highlights the importance of finding ways to specifically target malignant cells due to the highly conserved nature of these metabolic pathways.

Methionine

Methionine is an essential amino acid that is involved in a variety of metabolic pathways, such as methylation reactions, homocysteine synthesis, and the folate pathway [Figure 2]. This metabolite also cooperates with arginine and glutamine to promote polyamine and glutathione synthesis, respectively[190]. In the methionine pathway, methionine is converted to S-adenosyl methionine (SAM), which is critical for the methylation of histones, DNA, RNA, proteins, and various metabolites[191]. The loss of a methyl group converts SAM to S-adenosyl homocysteine (SAH), and subsequently homocysteine, which is ultimately metabolized to glutathione[192]. Methionine regeneration is supported by the metabolism of SAM through the salvage pathway[192] and through the re-methylation of homocysteine via intermediates in the folate pathway[193].

The role of methionine in malignant transformation and growth is not as well-studied as other metabolites, but its wide consumption in cancer cells suggests its importance[194,195]. In tumor-initiating cells, exogenous methionine is consumed at extreme rates, leading to pro-tumorigenic epigenetic modifications through methionine adenosyltransferase 2A (MAT2A), which metabolizes methionine to SAM to promote histone methylation[196]. In the presence of methionine, malignant cells activate c-MYC, leading to increased MAT2A activity and tumorigenic genome modifications[197]. On the other hand, tumor overexpression of nicotinamide N-methyltransferase (NNMT), which converts SAM to NAD^+ and 1-Methylnicotinamide, leads to increased NAD^+ levels, hypomethylation, and tumor progression[198], highlighting that altered methionine metabolism can drive oncogenesis in multiple ways.

In T cells, proper metabolic regulation of methionine and its derivatives is necessary for epigenetic reprogramming during activation and differentiation[199], as evidenced by increased expression of methionine transporters during antigen recognition[175]. However, dysregulated methionine metabolism by tumor cells alters the abundance of SAM and 5-methylthioadenosine (MTA)[200], both of which drive the methionine salvage pathway[201]. Increased abundance of SAM and MTA within the TIME are associated with T cell exhaustion and expression of inhibitory checkpoint markers[200]. These two metabolites decrease chromatin accessibility in $CD8^+$ T cells for genes involved in TCR signaling, lymphocyte proliferation and differentiation, and increase the accessibility of PD-1[200]. Together, these data indicate that tumor-derived alterations in methionine metabolism have a substantial impact on the anti-tumor immune functions of $CD8^+$ T cells, but much remains to be discovered.

Despite the limited studies in this field, two recent reports demonstrate that restricting tumor methionine increases $CD8^+$ T cell effector functions and overcomes resistance to anti-PD-1/PD-L1 treatment. The first study shows that dietary restriction of methionine reduces SAM levels in murine colorectal carcinoma tumors[202]. Mechanistically, SAM controls the expression of immune inhibitory markers PD-L1 and VISTA

through m^{6}A methylation, whereby the RNA-binding protein YTHDF1 enhances the translation efficiency of RNA containing m^{6}A methylation[202]. While anti-PD-1 treatment alone in mouse colorectal carcinoma tumors did not significantly alter tumor volume or CD8$^+$ T cell infiltration, depletion of YTHDF1 or restricting methionine in the diet synergized with anti-PD-1 treatment to significantly increase survival probability and CD8$^+$ T cell infiltration, while decreasing tumor volume[202]. Similarly, the second study found that methionine-dependent histone methylation regulates CD8$^+$ T cell anti-tumor activities. Methionine deprivation in CD8$^+$ T cells resulted in reduced H3K79me2 methylation and subsequent STAT5 expression[203], which is a critical transcription factor that maintains CD8$^+$ T cell effector functions[204]. *In vitro*, methionine supplementation increased CD8$^+$ T cell survival and IFNg and TNFa production, while inhibiting murine melanoma tumor growth[203]. The authors also found that SLC43A2 and SLC7A5 import methionine in malignant cells, but T cells are predominantly dependent on SLC7A5[203]. As such, genetic ablation of SLC43A2 in mouse melanoma cells restored CD8$^+$ T cell polyfunctionality and survival *in vitro*, and decreased tumor growth *in vivo*[203]. While anti-PD-1 treatment or pharmacological inhibition of SLC43A2 alone did not elicit significant anti-tumor effects, combination treatment synergistically increased CD8$^+$ T cell function and infiltration, and decreased growth of mouse melanoma and ovarian tumors[203]. These data demonstrate that resistance to anti-PD-1 treatment can be negated by restricting methionine availability and metabolism in tumors.

Taken together, the studies in this section have undoubtedly established that targeting amino acid metabolism is an efficacious way to improve the response to anti-PD-1/PD-L1 treatment. Targeting these metabolic pathways proves to be challenging because, unlike the immunosuppressive metabolites that have been discussed, amino acids are beneficial for both T cells and tumor cells. Therefore, therapeutic strategies have to promote amino acid supplementation in T cells but restriction in tumor cells, which is no easy feat. Despite these challenges, researchers have made great strides in pre-clinical settings towards identifying how to alter amino acid metabolism in a way that impedes ICB resistance.

LIPID METABOLISM

The TIME is enriched with various lipid classes[205-207], which is in contrast to other metabolites that are predominantly depleted. Lipids are ubiquitously important for structural support, energy supply, and signaling, making them essential for the malignant properties of tumors and for the proper function of anti-tumor immune cells. Specifically, cholesterol is indispensable for cell membrane integrity and facilitating cell-to-cell and intracellular signaling, while fatty acids (FAs) are the most abundant lipid intermediate, so they are more readily detectable and their role in cancer biology is better understood. Therefore, this section will highlight how tumor-mediated cholesterol and FA dysregulation within the TIME affects CD8$^+$ T cell function and anti-PD-1/PD-L1 resistance.

Cholesterol

Cholesterol serves as an important component in cellular membranes and regulates membrane fluidity and cell signaling through the formation of lipid rafts[208] [Figure 3]. Moreover, cholesterol is a precursor for steroid hormones, bile acids, and vitamin D[208]. Intracellular cholesterol levels are maintained through biosynthesis via the mevalonate pathway, which converts acetyl-CoA to cholesterol through a series of enzymatic reactions. Additionally, cholesterol is imported as low-density lipoproteins, which are small lipid-enclosed particles that facilitate the systemic transport and cellular import of cholesterol[209]. On the other hand, cholesterol is exported through ATP-binding cassette transporters[210]. Excess intracellular free cholesterol is converted to cholesteryl esters and stored in lipid droplets, which promote oncogenic signaling and cancer growth[211].

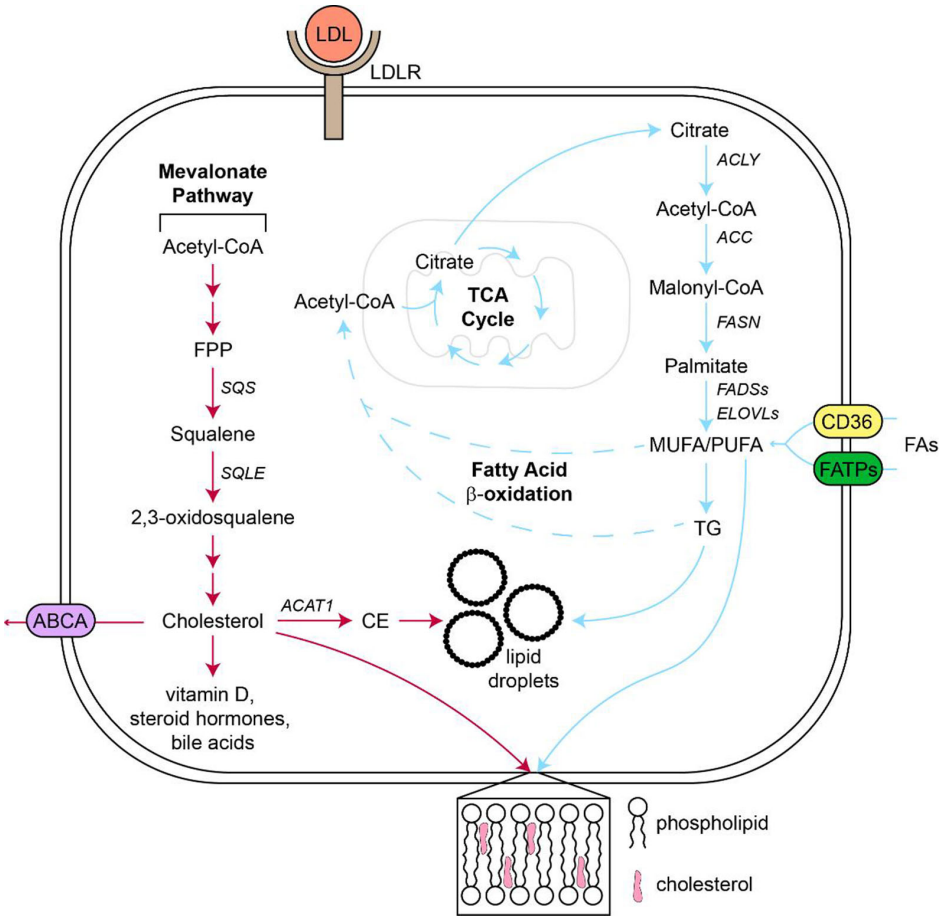

Figure 3. Diagram of cholesterol and FA metabolic pathways. Cholesterol is either imported as LDL through LDLR or it is synthesized through the mevalonate pathway. From there, cholesterol serves as a precursor to vitamin D, steroid hormones, and bile acids or it integrates into the cellular membrane to regulate membrane fluidity and cell signaling. Excess intracellular cholesterol is exported through ABCA or esterified to form CE, which are stored in lipid droplets. FAs are imported via CD36 and fatty acid transport proteins or synthesized through citrate from the TCA cycle. Palmitate, the initial FA that is formed, undergoes elongation and desaturation by ELOVL and FADS enzymes, respectively, to form a variety of FAs with varying chain lengths and degrees of unsaturation. FAs participate in energy metabolism through the FA b-oxidation pathway that generates acetyl-CoA to drive the TCA cycle. Similar to cholesterol, fatty acids are important components of cellular membranes via the formation of phospholipids and excess fatty acids are converted to TG and stored in lipid droplets. ABCA: ATP-binding cassette transporters; ACAT1: Acyl-CoA cholesterol acyl transferase 1; ACC: acetyl-CoA carboxylase; ACLY: ATP citrate lyase; ATP: adenosine triphosphate; CE: cholesteryl esters; ELOVL: elongation of very long chain fatty acids protein; FA: fatty acid; FADS: fatty acid desaturase; FATP: fatty acid transport protein; FASN: fatty acid synthase; FPP: farnesyl diphosphate; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; SQS: squalene synthase; SQLE: squalene epoxidase; TCA: tricarboxylic acid; TG: triglyceride.

Malignant cells utilize excess cholesterol to sustain their rapid growth and proliferation[212-214] and elevated intracellular cholesterol levels are maintained by increasing import and synthesis and decreasing export[215,216]. Altered cholesterol content in malignant cell membranes regulates apoptosis[217], proliferation, metastasis[218], and killing by cytotoxic T cells[219]. Cholesterol and its derivatives are also involved in various oncogenic signaling pathways and protein modifications[220]. Unsurprisingly, these metabolites are sequestered by tumor cells to promote malignant growth, and dysregulation of cholesterol in the TIME by tumor cells affects the cytotoxic functions of $CD8^+$ T cells.

There are multiple ways in which tumor cells directly alter cholesterol metabolism within the TIME to inhibit $CD8^+$ T cell function. Protein convertase subtilisin/kexin type 9 (PCSK9) is a secreted enzyme that

regulates cholesterol levels by facilitating the degradation of low-density lipoprotein receptors (LDLR)[221-224], which imports low-density lipoprotein cholesterol. Tumor-secreted PCSK9 promotes intratumoral accumulation of cholesterol[225], prevents LDLR and TCR recycling in $CD8^+$ TILs[226], and inhibits MCH-1 recycling on tumor cells[227], leading to immune evasion in multiple ways. Further, several reports demonstrate that intratumoral cholesterol accumulation promotes PD-L1 expression[228-231], thereby contributing to immune evasion. Mechanistically, cholesterol binds to the transmembrane domain of PD-L1 to stabilize cell surface expression[231]. Cholesterol-derived metabolites produced by malignant cells also dictate anti-tumor response. For example, cholesterol sulfate creates a chemical barrier within the TIME to prevent $CD8^+$ T cell infiltration[232]. Moreover, cholesterol sulfate-producing tumors are more resistant to ICB therapy[232] than tumors that do not produce this metabolite, demonstrating that targeting tumor-intrinsic cholesterol metabolism could enhance ICB outcomes.

In addition to cholesterol biochemical pathways regulating $CD8^+$ T cell function, mechanical forces driven by altered cholesterol levels within tumor cells also influence anti-tumor immune response. Cancer cells accumulate cholesterol within the cell membrane, leading to increased membrane fluidity, or "cell softening"[219]. This phenomenon is associated with cancer development and progression because cancer cell softening impairs the cytotoxic effects of T cells, leading to immune escape[219]. By reversing these effects and promoting cancer cell stiffening, increased T cell forces and actin accumulation at the immunological synapse enhance tumor killing[219]. Notably, cancer cell stiffening did not alter TCR signaling or cytokine production, demonstrating that these effects were purely through mechanical forces[219].

In T cells, maintaining a proper balance between membrane and intracellular cholesterol levels is important for development, activation, and effector functions. Cholesterol in the cell membrane is essential for the intricate formation of lipid rafts which regulate TCR signaling[233]. In TILs, several studies report that the allocation of cholesterol towards cell membrane formation instead of storage as cholesterol esters promotes anti-tumor activities. Pharmacologic inhibition in tumor cells and $CD8^+$ T cells of acyl-CoA cholesterol acyltransferase 1 (ACAT1), which promotes cholesterol esterification, inhibits cancer cell growth[234]. Similarly, another group found that RORa, a nuclear hormone receptor, promotes $CD8^+$ T cell membrane cholesterol accumulation by inhibiting cholesterol esterification, thus enhancing anti-tumor functions[235]. On the other hand, intracellular cholesterol accumulation in $CD8^+$ T cells due to cholesterol enrichment in the TIME leads to endoplasmic reticulum (ER) stress, which causes T cell exhaustion and increased expression of immune checkpoint markers[207]. Mechanistically, ER stress promotes upregulation of the ER stress sensing protein XBP1, which drives the expression of immune inhibitory markers, namely PD-1 and 2B4[207]. As a result, inhibiting XBP1 or reducing cholesterol in $CD8^+$ T cells or the TIME boosts the anti-tumor functions of $CD8^+$ T cells[207]. These studies demonstrate that shifting cholesterol away from intracellular stores towards membrane formation in T cells might be an effective therapeutic strategy to diminish resistance to ICB therapy.

Given the profound effect of tumor-derived cholesterol on $CD8^+$ T cell function, it is no surprise that targeting this altered metabolic pathway inhibits resistance to anti-PD-1 treatment. Building on the idea that allocating cholesterol towards cellular membranes in $CD8^+$ T cells is beneficial for the anti-tumor response, researchers found that pharmacologic inhibition of ACAT1 in combination with anti-PD-1 treatment synergistically reduced the growth of mouse melanoma tumors[236]. Further, slight anti-tumor effects were observed in four mouse tumor models following genetic ablation of PCSK9, but combination of genetic or pharmacologic inhibition of PCSK9 with anti-PD-1 resulted in robust synergistic effects to increase MHC-I expression and survival and reduce growth of murine melanoma and colorectal carcinoma tumors[227]. Another emerging target is squalene epoxidase (SQLE), which catalyzes one of the rate-limiting steps in

sterol synthesis [Figure 3]. Bioinformatics approaches have identified a negative correlation between SQLE expression in human pancreatic adenocarcinoma and immune cell infiltration and immunotherapy response[237], prompting the need for further validation of this potential target. While the intersection of tumor-mediated cholesterol metabolism and ICB response is not as robust as other metabolic programs, these recent studies hint at how this relationship can be exploited to overcome ICB resistance.

Fatty acids

Similar to cholesterol, FAs have a variety of cellular functions, including cell membrane formation through phospholipids, energy metabolism, and precursors for signaling lipids [Figure 3]. Intracellular FA abundance is regulated by import through CD36 or FA transport proteins and synthesis via fatty acid synthase (FASN) from acetyl-CoA or malonyl-CoA[238]. FAs undergo modifications to chain length to form long-chain FAs (LCFAs) or very long-chain FAs (VLCFAs) and saturation to form mono-, di-, and poly-unsaturated FAs. Saturation and chain length dictate FA function and their role in oncogenesis[239]. In energy metabolism, FAs are subject to fatty acid b-oxidation (FAO) in the mitochondria to generate FADH, NADH, and acetyl-CoA to fuel a variety of energetic processes[240].

The increased demand for FAs in malignant cells sustains their rapid proliferation by serving as an energy source via FAO and as an indispensable component for cell membrane formation. Moreover, certain FAs are important precursors for a variety of oncogenic signaling mediators[241-243]. To meet these metabolic demands, cancer cells will increase the uptake and synthesis of fatty acids, while also inducing lipolysis of neighboring adipocytes[244-248]. Continuous evidence is emerging that altered FA metabolism by tumor cells alters the lipidome in the TIME, contributing to $CD8^+$ T cell dysfunction. However, the effect of tumor-derived FA metabolic alterations on ICB resistance is not well-studied.

Malignant cells exploit the increased lipid availability in patients with obesity and remodel the TIME to inhibit $CD8^+$ T cell function and promote cancer growth. High-fat diet-induced obesity in multiple mouse models of cancer alters the metabolic profile of malignant cells to increase FA uptake and utilization and creates an immunosuppressive TIME that inhibits $CD8^+$ T cell infiltration and function[249]. Moreover, inhibiting obesity-induced metabolic rewiring in murine colorectal carcinoma tumors restores $CD8^+$ TIL function and increases anti-tumor immune function[249]. Mechanistically, researchers found that $CD8^+$ T cells in obesity-associated breast cancer tumors exhibit ligation of leptin and PD-1 to reduce effector functions through activation of STAT3, which promotes FAO and inhibits glycolysis[250]. PD-1 ligation also promotes FAO in T cells through upregulation of carnitine palmitoyltransferase 1A (CPT1A), an essential enzyme involved in FAO[251]. Further, obesity in mice, humans, and non-human primates leads to increased PD-1 expression and $CD8^+$ T cell exhaustion[252]. These data are consistent with the notion that $CD8^+$ T cells exhibit a shift from glycolysis to FAO as they become exhausted, highlighting the need to further explore targeting metabolic reprogramming as a way to reinvigorate $CD8^+$ T cells and abate ICB resistance.

Similar to obese models of cancer, non-obese models show that $CD8^+$ T cell function is inhibited by an overabundance of FAs within the TIME. In response to excess lipid content within the TIME, $CD8^+$ TILs exhibit increased intracellular lipid levels compared to peripheral $CD8^+$ T cells[205]. Exhaustion in $CD8^+$ TILs is characterized by the expression of CD36, which imports oxidized low-density lipoproteins, oxidized phospholipids, and long-chain fatty acids[205]. Increased uptake of oxidized low-density lipoproteins promotes lipid peroxidation in $CD8^+$ TILs, leading to decreased cytokine production and effector function[205]. Moreover, the accumulation of VLCFAs within the TIME drives the uptake of LCFAs in $CD8^+$ T cells, and instead of serving as an energy source, they promote mitochondrial dysfunction, lipotoxicity, and exhaustion[253]. Like cancer cells, immunosuppressive cells, such as Tregs, macrophages, and MDSCs,

rely heavily on exogenous FAs to sustain their increased rate of FAO[254-257]. In this regard, increased FA abundance within the TIME hinders $CD8^+$ T cell function, while benefiting malignant and immunosuppressive cells.

FAs are the building blocks for a variety of bioactive lipids, which are involved in signaling pathways. Tumor cells, and to a lesser extent CAFs[258], secrete the enzyme autotaxin (ATX) that converts ubiquitously available lysophosphatidylcholine (LPC) to the bioactive lipid lysophosphatidic acid (LPA)[259]. LPA modulates numerous signaling pathways through lysophosphatidic acid receptors 1-6 (LPAR1-6), which are present on a variety of cell types[259]. In malignant cells, the ATX/LPA axis also functions in an autocrine manner by promoting oncogenic signaling through LPAR1[260]. On $CD8^+$ T cells, tumor-derived LPA binds to LPAR6 and prevents tumor infiltration by inhibiting migration[260]. LPA also signals through LPAR5 on $CD8^+$ T cells to induce cytoskeletal dysfunction, immunological synapse malformation, and impaired cytokine secretion and intracellular calcium release[261-263]. LPAR5 signaling on $CD8^+$ T cells also induces an exhausted-like state by promoting metabolic stress through ROS production and ultimately impairing antigen-specific killing[264]. The recent development of a first-in-class ATX inhibitor demonstrated tumor growth inhibition in mouse models of breast cancer[265,266]. The safety of this compound was tested in Phase I clinical trials in 2021, where the drug was well-tolerated with no significant clinically adverse effects[266]. These promising results demonstrate the previously unexplored capacity to target ATX in solid tumors, with the future potential to combine this treatment with pre-existing ICB therapies.

There is very limited research on targeting FA metabolism in combination with anti-PD-1/PD-L1 therapy, but more evidence is emerging that supports this approach to overcome ICB resistance. Bioinformatics methods have identified that FASN expression in patients with bladder cancer, melanoma, and non-small cell lung carcinoma is linked to immune infiltration and ICB response[267,268]. Interestingly, ICB is more efficacious in obese patients with melanoma compared to non-obese patients[252,269-272]. While this may seem contradictory, obesity drives PD-1 expression on $CD8^+$ T cells, thus eliciting a more robust response. On the other hand, $CD8^+$ TILs in pancreatic adenocarcinoma exhibit increased expression of checkpoint inhibitors, but ICB therapy largely fails[273-275]. The variability in ICB response between cancer types prompts the need for a deeper understanding of the mechanisms that contribute to resistance. To further complicate things, under hypoxic and hypoglycemic conditions, pharmacologically enhancing FA catabolism in $CD8^+$ T cells promotes effector function[206]. Moreover, anti-PD-1 treatment, in combination with increased FA catabolism, synergistically reduced the volume of murine melanoma tumors and promoted anti-tumorigenic metabolic reprogramming in $CD8^+$ T cells[206]. These data suggest that under stressful conditions, i.e., oxygen and glucose depletion, increased FAO is required for $CD8^+$ T cell function, but this contradicts other studies that demonstrate a shift towards FAO promotes exhaustion.

Together, these research efforts have laid the groundwork to further characterize the intricate relationship between tumor-mediated cholesterol and FA metabolism and $CD8^+$ T cell function within the TIME. To date, it is not clear whether inhibiting cholesterol or FA metabolism is a viable treatment option to improve response to anti-PD-1/PD-L1 therapies. As new data emerges, researchers will have a better understanding of the tumor-specific cholesterol and FA metabolic programs that are exploited by cancer cells and if these can be targeted to prevent ICB resistance.

CONCLUSION

While ICB therapies have been an imperative advancement in cancer treatment, a majority of patients exhibit resistance, prompting the need for researchers to identify and target these resistance mechanisms. This review has provided a multitude of examples wherein tumor-intrinsic alterations to energy, amino

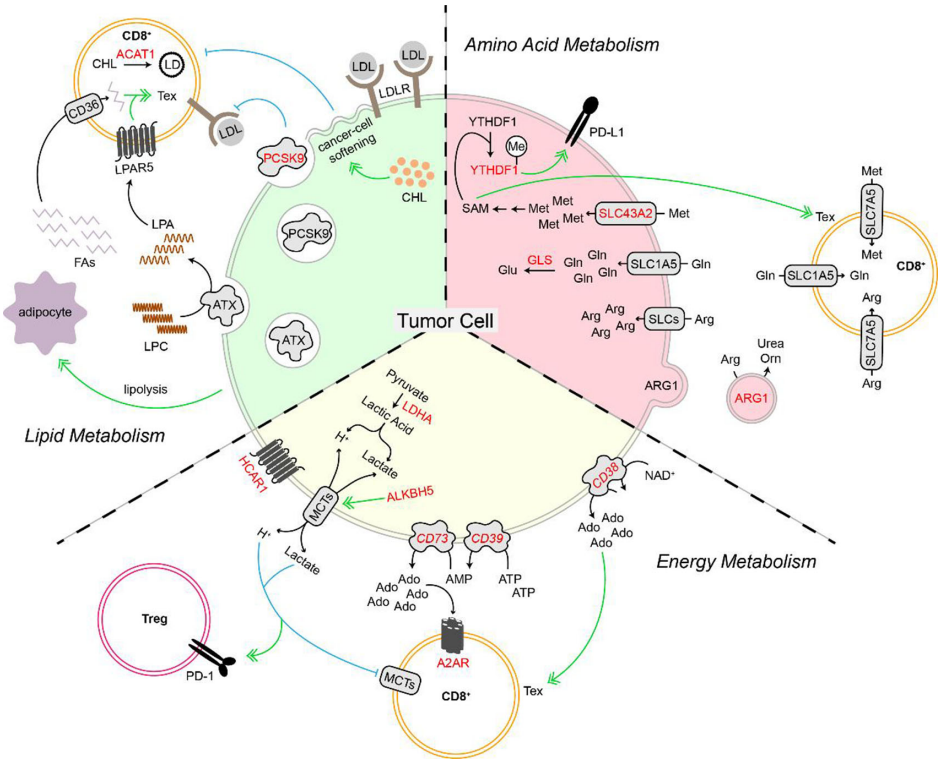

Figure 4. Summary schematic of how altered tumor-intrinsic energy, amino acid, and lipid metabolism drive $CD8^+$ T cell dysfunction and resistance to anti-PD-1/PD-L1 treatment. Targets in red are described in the previous sections and modulating these targets overcomes resistance to anti-PD-1/PD-L1 therapy. ACAT1: Acyl-CoA cholesterol acyl transferase 1; Ado: adenosine; ALKBH5: alkB homolog 5, RNA demethylase; Arg: arginine; ARG1: arginase 1; ATX: autotaxin; A2AR: adenosine A2A receptor; $CD8^+$: CD^+ T cell; CHL: cholesterol; FAs: fatty acids; Gln: glutamine; GLS: glutaminase; Glu: glutamate; HCAR1: hydroxycarboxylic acid receptor 1; LD: lipid droplet; LDHA: lactate dehydrogenase A; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; LPA: lysophosphatidic acid; LPAR5: lysophosphatidic acid receptor 5; LPC: lysophosphatidylcholine; MCT: monocarboxylate transporter; Me: methyl; Met: methionine; NAD^+: nicotinamide adenine dinucleotide; Orn: ornithine; PCSK9: proprotein convertase subtilisin/kexin type 9; PD-L1: programmed cell death ligand 1; PD-1: programmed cell death protein 1; SAM: S-adenosylmethionine; SLC: solute carrier; Tex: $CD8^+$ T cell exhaustion; Treg: T regulatory cell; YTHDF1: YTH N6-methyladenosine RNA binding protein F1.

acid, and lipid metabolism have a significant impact on $CD8^+$ T cell function and resistance to anti-PD-1/PD-L1 therapies [Table 1 and Figure 4]. In many of the studies presented here, anti-PD-1/PD-L1 therapy alone elicits limited anti-tumor effects but, when combined with targeting metabolic pathways, the response is significantly more robust. Nevertheless, there are a limited number of metabolism-targeting drugs that make it to the clinic because these pathways are highly conserved and not tumor-cell specific. As such, this warrants either unique ways to mitigate systemic effects, some of which have been provided in this review, or continued efforts to identify tumor-specific pathways. However, the extreme heterogeneity of the TIME, metabolome, and lipidome between cancer types necessitates large research efforts to uncover these distinct metabolic programs.

Future directions for the fields of immuno- and onco-metabolism are rooted in the utilization of metabolomic and lipidomic analyses to understand the metabolic landscape of cancer and develop efficacious cancer treatments. Taking a true multi-omics approach by incorporating proteomics, transcriptomics/spatial transcriptomics, and metabolomics/spatial metabolomics will greatly advance our understanding of targetable pathways, both within malignant cells and T cells. These methods are gaining more traction within the oncology research space and hopefully will be more widely utilized in the coming years.

Table 1. Tumor-intrinsic metabolic targets, the resulting metabolites, and the drug or compound used against the target that have been evaluated pre-clinically and/or clinically in combination with anti-PD-1/PD-L1 therapy

Target (metabolite)	Drug/Compound	Pre-clinical or clinical	Combination with anti-PD-1/PD-L1	Ref.
ALKBH5 (lactate)	ALK-04	Pre-clinical	Anti-PD-1	[42]
LDHA (lactate)	GSK2837808A	Pre-clinical	Anti-PD-1	[43]
HCAR1 (lactate)	3-OBA	Pre-clinical	Anti-PD-1	[44]
A2AR (adenosine)	CPI-444	Clinical	Anti-PD-L1	[84]
A2AR (adenosine)	DZD2269	Pre-clinical	Anti-PD-1	[85]
CD39 (adenosine)	IPH5201	Clinical	Anti-PD-L1	[89]
CD39 (adenosine)	IPH5201	Pre-clinical	Anti-PD-L1	[88-90]
CD39 (adenosine)	TTX-030	Clinical	Anti-PD-1	[91]
CD73 (adenosine)	MEDI9447 (oleclumab)	Clinical	Anti-PD-L1	[92,93]
A2AR (adenosine)	Nanoreactor	Pre-clinical	Anti-PD-1	[94]
CD39 (adenosine)	POM-1	Pre-clinical	Anti-PD-1	[95]
CD39 (adenosine)	ARL67156	Pre-clinical	Anti-PD-1	[96]
CD38 (NAD^+)	Anti-CD38 and RHein	Pre-clinical	Anti-PD-L1	[114]
ARG1 (arginine)	Vaccine	Pre-clinical	Anti-PD-1	[156]
ARG1/2 (arginine)	OATD-02	Pre-clinical	Anti-PD-1	[150,160-162]
ARG (arginine)	CB-1158	Clinical	Anti-PD-1	[163-165]
SLC1A5 (glutamine)	V-9302	Pre-clinical	Anti-PD-L1	[183]
Glutamine-utilizing enzymes (glutamine)	JHU083	Pre-clinical	Anti-PD-1	[184]
YTHDF1 (methionine)	Short-hairpin knockdown of YTHDF1	Pre-clinical	Anti-PD-L1	[202]
SLC43A2 (methionine)	BCH	Pre-clinical	Anti-PD-L1	[203]
ACAT1 (cholesterol)	CI-1011	Pre-clinical	Anti-PD-1	[236]
PCSK9 (cholesterol)	AMG-145 and D10335	Pre-clinical	Anti-PD-1	[227]

ACAT1: Acyl-CoA cholesterol acyl transferase 1; ALKBH5: alkB homolog 5; ARG1: arginase 1; A2AR: adenosine A2A receptor; HCAR1: hydroxycarboxylic acid receptor 1; LDHA: lactate dehydrogenase A; PCSK9: proprotein convertase subtilisin/kexin type 9; PD-L1: programmed cell death ligand 1; PD-1: programmed cell death protein 1; SLC: solute carrier; YTHDF1: YTH N6-methyladenosine RNA binding protein F1.

PERSPECTIVES

In recent years, immense strides have been made in studying the intersection of metabolism, cancer, and the immune system. In addition to the metabolites and pathways covered in this review, there are a plethora of others waiting to be linked to $CD8^+$ T cell dysfunction and ICB resistance. For example, other amino acids and lipid classes, metabolites produced by the gut microbiome, and a closer look at the metabolites associated with oxidative phosphorylation and ATP production. Moreover, there is much to uncover about how tumor-derived metabolic alterations affect other immune and non-immune cell types. Continued research efforts in this field will provide a more comprehensive understanding of tumor-intrinsic metabolic alterations and reveal nuanced ways to target tumor metabolism and overcome resistance to ICB therapies.

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Cancer Drug Resistance

Review

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Emerging resistance *vs.* losing response to immune check point inhibitors in renal cell carcinoma: two differing phenomena

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Abstract

The introduction of immune checkpoint inhibitor (ICI) has revolutionized the treatment of metastatic renal cell carcinoma (mRCC) and has dramatically improved the outcomes of patients. The use of monotherapy or combinations of ICIs targeting PD-1/PD-L1 and CTLA-4, as well as the addition of ICIs with tyrosine kinase inhibitors, has significantly enhanced the overall survival of mRCC patients. Despite these promising results, there remains a subset of patients who either do not respond to treatment (primary resistance) or develop resistance to therapy over time (acquired resistance). Understanding the mechanisms underlying the development of resistance to ICI treatment is crucial in the management of mRCC, as they can be used to identify new targets for innovative therapeutic strategies. Currently, there is an unmet need to develop new predictive and prognostic biomarkers that can aid in the development of personalized treatment options for mRCC patients. In this review, we summarize several mechanisms of ICI resistance in RCC, including alterations in tumor microenvironment, upregulation of alternative immune checkpoint pathways, and genetic and epigenetic changes. Additionally, we highlight potential strategies that can be used to overcome resistance, such as combination therapy, targeted therapy, and immune modulation.

Keywords: Renal cell carcinoma, immunotherapy, immune checkpoint inhibitors, primary resistance, acquired resistance, immune exhaustion markers, immunosuppressive tumor microenvironment

INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 3% of all adult malignancies[1]. The incidence of RCC has been steadily increasing with 81,800 new cases estimated to be diagnosed in the United States in 2023[2]. The 5-year relative survival rate has increased over time, with 72% survival for patients with locoregional disease, but the survival for metastatic RCC is still poor: 15%[2]. There are different types of RCC with diverse clinical and epigenetic characteristics, of which clear cell type is the most common variant (80%), followed by papillary (15%) and chromophobe (3%-5%) histological variants[3].

The main therapeutic options in metastatic RCC (mRCC) were interferons and interleukins till 2006. Significant advancements have been made in the field of RCC with the development of vascular endothelial growth factor receptors (VEGFR) inhibitors and mammalian target of rapamycin (mTOR) inhibitors[4-6]. RCC has been recognized as an immunosuppressive disease. With the advent of novel immune checkpoint inhibitor (ICI) targeting programmed death-1 (PD-1), programmed death ligand-1 (PD-L1) and cytotoxic T-lymphocyte associated protein-4 (CTLA-4), the treatment paradigm of RCC has changed tremendously[4,7,8].

Currently, ICI monotherapy and ICI combination with TKI/ICI are the standard of treatment in advanced RCC, which have significantly improved the survival of treatment-refractory mRCC patients. Nivolumab, an anti-PD-1 antibody, was approved for use in mRCC in 2015 based on the overall survival benefit seen in the CheckMate 025 trial. The objective response rate (ORR) with nivolumab was 25% compared to 5% with everolimus[9]. Following this, based on the results of the CheckMate 214 trial, the combination of nivolumab and ipilimumab was approved for use in treatment-naive intermediate and poor-risk mRCC patients [International Metastatic Renal Cell Carcinoma (IMDC) risk stratification criteria][10]. The combination has resulted in an improvement in overall survival, with an ORR of 42% and a complete response rate of 11% over a span of 5 years[11]. Several VEGFR-TKIs have shown survival advantages in mRCC when used in combination with the ICI. The combination of pembrolizumab (PD-1 inhibitor) and axitinib (VEGFR inhibitor) (Keynote-426), nivolumab and cabozantinib (CheckMate 9ER), lenvatinib and pembrolizumab (CLEAR trial), avelumab (PD-L1 inhibitor) and axitinib (JAVELIN Renal 101) are approved as first-line options for patients with mRCC[12-15]. All these treatments have resulted in a superior response rate and progression-free survival (PFS) compared to comparator, sunitinib.

Although these treatments have shown long-term clinical benefits in a large fraction of patients, some patients have progressive disease as the best response, consistent with primary resistance. Some patients progress after responding for a certain period, reflecting acquired resistance to these systemic treatment options. All these treatments have improved the outcomes in mRCC; however, these are limited by the innate and acquired resistance that emerges. The cancer-immunity cycle, which involves the development of neoantigens, antigen presentation, T-cell responses, and the recognition and destruction of cancer cells, describes the inherent immune biology of cancer and resistance to ICIs[16]. Understanding the mechanisms behind the resistance is needed to develop therapeutic strategies to overcome it and thus maximize therapeutic efficacy. In this review article, we mention the mechanism of action of ICI, highlight the mechanisms of resistance to ICI, and the potential approaches to overcome resistance to ICI in mRCC.

Mechanism of action of immune checkpoint inhibitors

The mechanism of resistance to ICI is not completely understood. To understand the biology behind the resistance formation, an adequate understanding of the mechanism of action of immunotherapy is necessary. The major histocompatibility complex (MHC)/antigen on the antigen-presenting cells (APCs) interacts with the T cell receptors (TCR) and activates the T cells which lead to a cascade of events involving

stimulatory and inhibitory signals[17]. Upon activation, the T cells release interferon-gamma (IFN-γ), which promotes cytotoxicity and results in the upregulation of PD-L1 expression in the tumor cells. The PD-1, which is expressed on the activated T cells, interacts with the PD-L1, resulting in the inhibition of the antitumor response by the T cells[18]. Similarly, the interaction of CTLA-4 expressed on the T cells with its ligands CD80/CD86, B7 on APC prevents the stimulation, proliferation, and activation of T cells, thus diminishing the immune response[19,20]. The above is responsible for T cells being anergic and relatively inactive against certain cancers like RCC and melanoma. Expression of other coinhibitory receptors, such as TIM-3 and LAG-3, leads to T-cell exhaustion[21]. ICIs targeting PD-1/PD-L1, and CTLA-4 can facilitate T-cell activation and overcome anergy and thus improve the antitumor immune response[7] [Figure 1].

Mechanism of resistance to immunotherapy in RCC

The resistance to ICI occurs due to the complex and evolving interactions between the immune system and cancer cells. Several patient factors, tumor microenvironment factors, and oncogenic signaling pathways play an essential role in the development of resistance to systemic therapies. The resistance to immunotherapy can be classified broadly into two categories: (a) primary resistance, in which patients will have progressive disease as best response to immunotherapy; and (b) acquired resistance, in which patients will respond to immunotherapy for some time and eventually have progression of the disease. This could occur in two ways: (a) resistance formation while on the drug (acquired resistance) and (b) progression of disease after a long treatment-free interval, which is uniquely seen after discontinuation of therapy upon the development of immune-related adverse events (IRAE) (we call it "loss of response") [Figure 2]. There is no specific consensus regarding the timeline of the development of the acquired resistance[21]. High tumor mutational burden (TMB) and high neoantigen expression have been associated with a more consistent response to ICI[22]. Resistance to ICI therapy can be due to a defect in any of the steps explained in the mechanism of action of ICI. It could be due to insufficient production or impaired function of antitumor T-cells, or lack of adequate memory T-cell formation. Several patient factors, tumor microenvironment (TME) factors, oncogenic pathways, and immune checkpoints impact the creation and sustainment of an antitumor microenvironment.

Tumor microenvironment

TME in RCC is composed of several factors, such as extracellular matrix, immune cells, stromal cells, aberrant blood vessels, cytokines, and growth factors, which affect the growth, development and progression of the tumor and the treatment response. RCC is one of the tumors which has a high immune microenvironment composed of T cells. Increased proportion of the T regulatory cells (Tregs) in the TME is associated with an immunosuppressive environment in several malignancies[23]. Tregs suppress the T effector (T-eff) cells through inhibitory cytokines such as transforming growth factor (TGF)-β, interleukin (IL)-10, direct cytotoxicity through perforins/granzyme, promotion of T cell exhaustion and thus prevent the tumor-specific immune response which leads to resistance to ICI[24-26]. The proportion of Tregs has predictive and prognostic values[27-29]. In a study by Griffiths *et al.*, a high frequency of Tregs in the peripheral blood of RCC patients was found to be associated with reduced survival[30].

Myeloid-derived suppressor cells (MDSCs) in the TME are potent suppressors of various T cell functions, which facilitate tumors to evade immune responses. They regulate T cell proliferation, induce T cell apoptosis, and are involved in the inhibition of MHC class II proteins through several mechanisms such as arginase and nitric oxide production[31]. Tumor-associated factors such as TGF-β, platelet-derived growth factors (PDGFs), and Interleukins (IL-3, IL-6, IL-10) induce the production of reactive oxygen species (ROS) by the MDSCs. ROS has been postulated to be one of the mechanisms through which MDSCs inhibit the cytotoxicity of T cells and create an immunosuppressive environment[32]. In one of the studies, the depletion of MDSCs reinstituted IFN-γ production and T-cell proliferation in RCC[33]. Thus, MDSCs, along

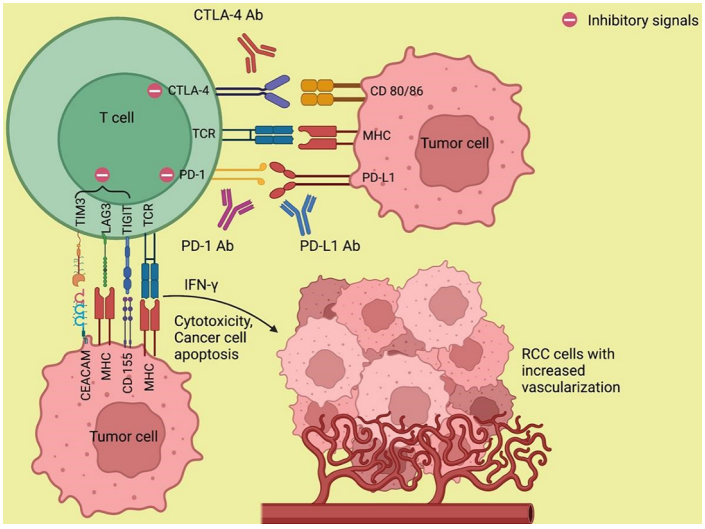

Figure 1. Mechanism of action of immune checkpoint inhibitors. The monoclonal antibodies against PD-1/PD-L1 and CTLA-4 abolish the T-cell inhibitory responses that are stimulated by the interaction of PD-1 with PD-L1 and CTLA-4 with its ligand CD 80/86 and thereby enhance antitumor immunity. Ab: Antibody; CD 80/86: cluster of differentiation 80/86; CEACAM: carcinoembryonic antigen-related cell adhesion molecule; CTLA-4: cytotoxic T lymphocyte-associated protein 4; IFN-γ: interferon-γ; LAG3: lymphocyte-activation gene 3; MHC: major histocompatibility complex; PD-1: programmed death-1; PD-L1: programmed death ligand-1; RCC: renal cell carcinoma; TCR: T cell receptor; TIGIT: T cell immunoglobulin and ITIM domain; TIM3: T cell immunoglobulin and mucin domain 3. (Figure credits: Roy AM).

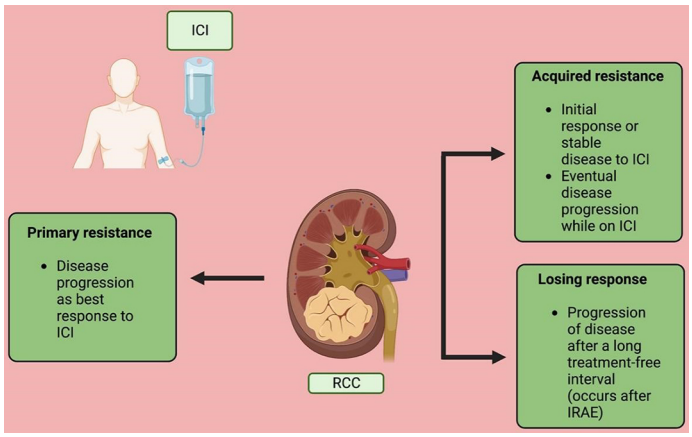

Figure 2. Types of resistance. ICI: Immune checkpoint inhibitor; IRAE: immune-related adverse events; RCC: renal cell carcinoma. (Figure credits: Roy AM).

with Tregs, create an immunosuppressive tumor environment that resists the activity of ICI.

Another component of the TME that impacts the response to ICI is tumor-associated macrophages (TAMs). During tumor development and progression, macrophages are recruited into the TME and differentiate into mature forms which aid in tumor progression. Macrophages are classified into M1 and M2 subtypes[34]. M1 macrophages are associated with inflammatory responses by secreting pro-inflammatory cytokines such as IL-6, IL-12, IL-23, and tumor necrosis factor-α (TNF-α). Nevertheless, the M2 macrophages, especially M2d, employ anti-inflammatory and pro-tumorigenic activities[35,36]. Studies have shown that TAMs promote tumor growth, invasion, and metastasis by altering the TME, enhancing angiogenesis and resulting in immune evasion and thus therapeutic resistance[37-39]. TAM also has prognostic

implications; malignancies with high TAM density have been shown to have poor disease-free survival (DFS) and overall survival (OS)[40,41].

Several cytokines in the TME also play a major role in immunoregulation. The cytokines CCL5, CCL17, CCL22, CXCL8, and CXCL12 promote immunosuppression by recruiting Tregs and MDSCs to the TME[42]. Similarly, cytokines also promote antitumor effects, as exhibited by the increased recruitment of cytotoxic T cells (CTLs) to the TME by the cytokines CXCL9 and CXCL10[43]. Several growth factors, such as TGF-β, Vascular endothelial growth factor (VEGF), emulate immunosuppression by upregulating Tregs and inhibiting the CTLs which impacts the response to ICI[44,45]. By priming the vascular endothelium to allow for the extravasation of Tregs and resistance to the migration of CTLs to the TME, VEGF contributes to immune evasion and promotes rapid tumor growth and progression, while also resulting in resistance to immune checkpoint inhibitors (ICIs).

Single-cell transcriptomic studies and paired single-cell T-cell receptor sequencing have enabled us to comprehend the immune cell composition and the role of T-cell clonotype expansion in response to ICIs[46,47]. The study by Braun *et al.* shows progressive immune dysfunction in metastatic RCC, as evidenced by a higher proportion of exhausted T-cells and immunosuppressive M2 macrophages. Additionally, it was observed that TAMs exhibit reduced production of inflammatory cytokines in advanced stages. While their study did not find a higher T-cell exhaustion/TAM interaction signature to be predictive of response to ICI, it was associated with poor overall survival[46]. The role of CD8 T-cells in the clinical outcomes of mRCC patients treated with ICI remains controversial. Several studies have linked the infiltration of CD8 T-cells in the tumor microenvironment of RCC patients to a poorer prognosis[48,49]. Exploratory data from the JAVELIN RENAL 101 trial revealed an association between high CD8 T cell infiltration and poor PFS in mRCC patients treated with sunitinib[50]. This trend was not evident among patients treated with the combination of avelumab + axitinib. In another study by Voss *et al.*, no association was found between CD8 T cell density and clinical response to ICI[51]. Hence, based on the current literature, we are unable to definitively establish a correlation between CD8 T cells and treatment response or resistance to ICI therapy, although such a possibility warrants consideration.

Oncogenic pathways and antigen presentation

Impaired antigen presentation could be associated with ICI resistance. The loss of MHC class molecules results in immune evasion and is known as a mechanism of acquired resistance to ICI. Loss of function of B2-microglobulin results in defective transport of the MHC-1 class molecules and selective downregulation of MHC-1 is associated with resistance to ICI[52]. This was evident in a study led by Zaretsky *et al.*, which showed that a truncated mutation in the gene encoding the antigen-presenting protein B2-microglobulin led to the loss of surface expression of MHC-1 molecules and resulted in acquired resistance to PD-1 blockade immunotherapy in melanoma[53].

Several signaling pathways mediate immune responses and contribute to the resistance to immunotherapy. JAK/STAT receptor upregulates MHC-1 expression through IFN-γ signaling which enhances antigen presentation. It also recruits immune cells to the TME and has antiproliferative effects on tumor cells and also enhances apoptosis[54]. It was noted that the loss of function mutations in the genes encoding JAK1 or JAK2 resulted in a lack of response to IFN-γ, affects the antiproliferative effects on tumor cells, and thereby affects the response to ICI therapy in malignancies[53]. Simultaneously, JAK/STAT receptors also increase PD-L1 expression on tumor cells through interferon regulatory factor 1 (IRF1) which grants resistance to tumor cells to the innate immune system of the body[55]. In the setting of ICI targeting PD-1, PD-L1 amplification has shown an improved response to treatment in some malignancies[56]. Acquired JAK/STAT

mutation results in loss of IFN-γ signaling and leads to resistance to ICI treatments through the inability to upregulate MHC-1 and PD-L1 expression[53,57].

The mitogen-activated protein kinase (MAPK) pathway has been shown to have a significant role in immune evasion. Increased MAPK signaling impairs the recruitment and function of T cells, resulting in immune evasion. MAPK signaling is also involved in the proliferation, apoptosis, and motility of tumors[58]. It has been shown that MAPK inhibitors promote cytotoxicity by enhancing IFN-γ signaling, MHC-1 expression, tumor-infiltrating lymphocytes and upregulating PD-L1 expression[59].

The Wnt/β-catenin pathway is overexpressed in many cancers. It takes part in tumorigenesis through the maintenance of cancer stem cells and metastasis and also affects cellular immune regulation[60]. Spranger *et al.* demonstrated that the activation and overexpression of the Wnt/β-catenin pathway are associated with resistance to anti-PD-L1 and anti-CTLA-4 monoclonal antibody therapies through T cell exclusion from the microenvironment. They also showed that activation of the WNT/β-catenin signaling pathway results in the absence of a T-cell gene expression signature[61]. Loss of phosphatase and tensin homolog (PTEN) leads to the expression of VEGF and activation of the phosphatidylinositol 3-kinase (PI3K) pathway, which aids in tumorigenesis. PTEN deletion also leads to the recruitment of immunosuppressive cytokines in the TME, decreased infiltration of CTLs into tumor sites and has shown inferior outcomes with PD-1 therapy[62].

Immune checkpoints

PD-1 has been well-known as a marker of T-cell exhaustion. The other relevant immune checkpoints include CTLA-4, T-cell immunoglobulin mucin-3 (TIM-3), and lymphocyte-activation gene 3 (LAG-3), B and T lymphocyte attenuator (BTLA), T-cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT). As mentioned earlier, the exhausted T-cells in advanced RCC exhibit several exhaustion markers, such as PD-1, TIM-3, TIGIT, and CTLA-4[46,47]. Cai *et al.* demonstrated that in RCC, TIM-3 expression was associated with infiltration of dysfunctional CTCLs and blockage of TIM-3 pathway in enhanced IFN-γ production and enhanced antitumor immunity[63]. It has been demonstrated that the co-expression of these inhibitory immune checkpoints such as PD-1, TIM-3, CTLA-4, and LAG-3 is associated with the progression of lung cancer[64]. Expression and upregulation of multiple immune checkpoints lead to T-cell exhaustion and have been linked with acquired anti-PD-1 and anti-CTLA-4 resistance in mouse models and clinically in several malignancies[64-66]. Persistence of antigen or chronic exposure to antigen results in over-expression of these inhibitory immune checkpoints and leads to impaired effector T-cell function. Studies have shown that high expression of PD-1 leads to excessive T cell exhaustion and results in poor response to ICI, although tumors with low/intermediate PD-1 expressing CD8 T cells can be stimulated with ICI and lead to therapeutic response. Therefore, a threshold level of PD-1 expression on the intratumor CD8 T-cells can predict treatment response in at least some cancers[67]. In addition to its role in the T-cell compartment, CTLA-4 is also reported to play a role in T-cell priming, peripheral tolerance, thymic development, and various other immunological functions that hold potential for therapeutic applications[68].

Impaired T-cell memory

At least ICI-ICI combination has long-term durable responses as evidenced by the extended follow-up of Checkmate -214, where the combination immunotherapy ipilimumab + nivolumab remained efficacious even after 4 years[11]. The effector CD8 T-cells play a pivotal role in the response to ICI as explained. A minority of the effector T cells transform into memory T cells inducing memory and remain inactive until repeated exposure to the antigens, thought to be the reason behind durable responses to dual CTLA-4/PD-1 blockade. It is evident that if the formation of the memory T cells is impaired, it can lead to the loss of response to ICI and will also lead to the development of acquired resistance[69,70]. In a study by Ribas *et al.*, it was demonstrated that patients who had poor response to anti-PD-1 therapy had significantly lower tumor-

associated memory T cells compared to those who responded[69].

Other mechanisms of resistance

Given that RCC is a hypervascular tumor, the excessive demand and inadequate supply of nutrients lead to hypoxia, which plays a role in generating an immunosuppressive microenvironment for the tumor to facilitate its progression. This acts as a vicious cycle as hypoxia upregulates genes involved in angiogenesis, cellular proliferation and it aids in the recruitment of immunosuppressive T-regs, MDSCs and accelerates the polarization of macrophages to TAMs, leading to the inhibition of the production and function of CD8 T-cells[71]. In addition, the release of hypoxia-induced-factors (HIF) 1 and 2 leads to overexpression of inhibitory checkpoints CTLA4, TIM3, and LAG3 through the generation of VEGF, leading to a more complex tumor microenvironment and thus resistance to ICI[72].

The association of gut microbiome in the response to ICI has been revealed. Analysis of the fecal microbiota sample showed a significant relative abundance of specific species of microbes (Ruminococcaceae family) in the anti-PD-1 responders in melanoma. Enhanced systemic and antitumor immunity was observed in anti-PD-1 responders with favorable gut microbiota. In addition, this response was observed in germ-free mice with fecal transplants from responders. The tumors of mice that received the fecal microbiota transplant from the anti-PD-1 responders had higher CD8 T-cell density compared to the mice that received the transplant from the non-responders[73]. The utilization of antibiotics altering the gut microbiota has been shown to have a negative impact on the overall survival of several malignancies including RCC[74-76]. Lalani *et al.* have demonstrated that the use of antibiotics 8 weeks before to 4 weeks after the initiation of immunotherapy has been associated with lower ORR and poor clinical outcomes with significantly reduced PFS[74]. Data are lacking regarding the duration and timing of antibiotics that could impact the clinical outcomes of ICI. Increased stress-related β-adrenergic signaling has been shown to create an immunosuppressive TME and promote tumor growth[77].

The role of cytoreduction nephrectomy in relation to ICI response remains relatively unestablished. Currently, there are no phase III clinical trials that have comprehensively evaluated the impact of cytoreductive nephrectomy on ICI response in RCC. A meta-analysis indicated that cytoreductive nephrectomy did not exhibit a significant association with the response to ICI treatments[78].

The main mechanisms of resistance to ICIs in RCC are discussed in Table 1. All the above factors contribute to the response of tumors to ICI, and by understanding the molecular mechanisms behind the resistance to ICI, we can identify potential targets for therapeutic interventions to sensitize tumors to ICI in RCC. The main mechanisms of resistance to ICIs and major trials addressing the mechanisms of resistance in RCC are given in Table 1.

Strategies to overcome resistance to immune checkpoint inhibitors

As mentioned above, the mechanisms of resistance to ICI are diverse, including the intra (PD-1 expression, TMB, *etc.*) and extra-tumoral factors (T-cell types and their infiltration, T-cell activation status, TAM, *etc.*). The main strategies that are utilized to overcome the primary resistance and prevent the acquired resistance to ICI are depicted below.

Combination of treatments

One of the main strategies that is used to overcome resistance to ICI is a combinatorial approach. The combination of anti-PD-1 nivolumab and anti-CTLA-4 ipilimumab ICIs has already been approved and used with excellent clinical benefit in mRCC[10]. The clinical benefit was observed to be higher in the combination of ICIs compared with ICI monotherapy. The ORR with nivolumab monotherapy was 25%

Table 1. Mechanisms of resistance in renal cell carcinoma

Mechanisms of resistance	Main components involved in resistance	Major trials addressing resistance to ICI
Tumor microenvironment	CD8+ T-cells	JAVELIN RENAL 101 exploratory analysis[50], NCT03013335
	T-regulatory cells	
	Myeloid-derived suppressor cells	
	Tumor-associated macrophages	NCT03013335
	Memory T-cells	
	Cytokines	
Oncogenic pathways	IFN-γ signaling	NCT03010176
	MAPK pathway	
	Wnt/β-catenin pathway	
Immune checkpoints	PD-1	TITAN-RCC, TiNivo-2 (NCT04987203)
	PD-L1	CONTACT-03
	CTLA-4	NCT03849469, TITAN-RCC
	TIM3	NCT03652077, NCT02608268
	LAG3	NCT02996110, NCT02996110, NCT03849469, NCT00351949
	BTLA	
	TIGIT	NCT03119428
Other factors	Hypoxia	NCT03634540, NCT04195750, NCT03634540
	Gut microbiome	NCT03829111, GETUG-AFU 26 NIVOREN multicenter phase II study[79]

BTLA: B and T lymphocyte attenuator; CTLA-4: cytotoxic T lymphocyte-associated protein 4; ICI: immune checkpoint inhibitor; IFN-γ: interferon-γ; LAG3: lymphocyte-activation gene 3; MAPK: mitogen-activated protein kinase; PD-1: programmed death-1; PD-L1: programmed death ligand-1; RCC: renal cell carcinoma; TIGIT: T cell immunoglobulin and ITIM domain; TIM3: T cell immunoglobulin and mucin domain 3.

(refractory RCC population) and that of the ipilimumab/nivolumab combination was 42% (treatment naïve RCC population). Similarly, better OS was observed with the combination therapy when used in the first-line setting; OS of ipilimumab/nivolumab therapy in intermediate and poor-risk mRCC patients was 48.1 months (55 months in the ITT) and that of nivolumab monotherapy was 25 months (second line)[9-11]. CTLA-4 inhibition has been involved in the priming of T cells, depleting the immunosuppressive Tregs in the TME and anti-PD-1 has a role in reversing T cell exhaustion[80].

Another strategy that is used to tackle acquired resistance to ICI is rechallenging the tumors after losing the response to ICI. One of our mRCC patients had a response for 18 months and single-agent pembrolizumab was discontinued for cutaneous IRAE. This response was maintained off therapy for another 18 months and then had progressive disease. We rechallenged the patient with single-agent pembrolizumab and observed a partial response again prior to a treatment holiday. This was consistent with loss of response rather than resistance formation. Although we have observed this in clinical practice, the accurate mechanism behind response on rechallenge is not completely understood. In metastatic melanoma, rechallenging patients with primary and secondary resistance to ICI have shown to have better therapeutic outcomes. In addition, the escalation of treatment (ICI combined with additional agents) has been shown to have higher response rates in melanoma[81]. A phase II study of salvage ipilimumab/nivolumab by Atkins *et al.* in mRCC has shown only modest benefit and is associated with increased side effects[82]. The benefit of Salvage ipilimumab and nivolumab in mRCC patients with primary and acquired resistance was analyzed in the TITAN-RCC trial[83]. They observed that half of the patients who received salvage ipilimumab/nivolumab for progression of the disease had clinical benefits (PR/CR 18% and SD 30%). This was irrespective of the timing of resistance development. In the recently published preliminary results of the phase III CONTACT-03

multicenter trial, patients with locally advanced or mRCC who progressed on prior ICI were randomized to receive atezolizumab and cabozantinib *vs.* cabozantinib alone. The addition of atezolizumab to cabozantinib did not lead to improved clinical outcomes. The median PFS in the atezolizumab and cabozantinib arm was 10.6 months (95%CI 9.8-12.3) compared to 10.8 months (95%CI 10.0-12.5) in the cabozantinib arm (HR = 1.03, P = 0.78). Similarly, the median OS in the combination arm was 25.7 months (95%CI 21.5-not evaluable) and was not evaluable in the cabozantinib arm (HR = 0.94, P = 0.69). The toxicities were higher in the combination arm, with 17 deaths compared to 9 deaths in the cabozantinib arm[84]. Another ongoing randomized phase III trial, TiNivo-2 trial, compares the effect of the combination of nivolumab and tivozanib with tivozanib monotherapy in patients who progressed on at least one line of ICI (NCT04987203)[85]. More biomarker studies are required to effectively understand the role of rechallenge/ escalation strategy in mRCC without increasing the immune-related toxicities[86]. It would be ideal if such studies incorporated a measure of the immune status, which could ideally include factors of tumor (real-time), T-cell/ TAM, T-cell receptor repertoire, and other relevant host factors.

ICIs are combined with several targeted agents to enhance their efficacy. Several combinations of TKIs are approved in combination with ICIs, as mentioned previously. The addition of cabozantinib to nivolumab in the first-line setting in mRCC, as noted in the Checkmate 9ER, resulted in prolonged overall survival than that was observed with sunitinib alone. The OS with the combination was 49.5 months and that of sunitinib was 35.5 months. The ORR was significantly higher with the combination (55.7% with cabozantinib + nivolumab: 28% with sunitinib), which points to the fact that the combination of TKIs and nivolumab has an additive effect[9,14,87]. VEGF inhibitors have been studied in combination with ICIs and are shown to reverse resistance to ICI to a certain extent by regulating the immunosuppressive TME[88]. VEGF induces various changes in the TME, including the upregulation of PD-L1, PD-1, and CTLA-4 expression on dendritic cells and other immune cells, leading to immune exhaustion[89]. VEGF also elevates the proportion of MDSCs and T-regs in the TME, fostering an immunosuppressive environment[90,91]. Moreover, it impedes the differentiation of CD8+ T cells and exerts an inhibitory impact on effector T-cells[92]. When TKIs, which inhibit VEGF, are combined with ICIs, they reverse these immunosuppressive effects of VEGF, thereby enhancing T-cell priming and promoting the cytotoxic activities of immune cells[93].

The combination of VEGF inhibitor bevacizumab and anti-PD-L1 atezolizumab has been studied in mRCC, and it was found that the combination increases CD8 T-cell density in the tumors and aids in antigen-specific T-cell migration[94]. Unfortunately, this clinical trial was negative and failed to demonstrate meaningful improvement in survival.

Targeting TME components

As immunosuppressive TME is a major factor causing resistance to ICIs, several components of the TME such as MDSCs, Tregs, and cytokines can be targeted to potentially reverse the resistance. As mentioned above, several cytokines play a role in recruiting immunosuppressive cells and CTLs into the TME. Epigenetic silencing of CXCL9 and CXCL10 expression can lead to resistance to ICI by reduction of the tumor-infiltrating lymphocytes[43]. In an ovarian cancer model, epigenetic modulator therapy reversed the suppression of these cytokines and improved response to ICIs[95]. In metastatic urothelial cancer, the inhibition of TGF-β is found to be associated with improved response to ICIs[96].

Targeting immune exhaustion markers

The overexpression of the immune exhaustion marker such as TIM-3, and LAG-3 leads to resistance to ICI therapy as mentioned above. TIM-3 has been reported as a predictive biomarker of ICI response in RCC. In RCC, co-expression of TIM3 and PD-1 correlated with large tumor size, aggressive phenotype, lower PFS, and OS and showed a higher risk of relapse[97]. Therefore, targeting these exhaustion markers alone or in

combination with PD-1 can lead to better clinical response. There are several ongoing clinical trials studying the effect of anti-TIM3 antibodies alone and in combination with anti-PD-1/PD-L1 antibodies in advanced solid tumors and hematological malignancies (NCT02817633, NCT03099109). The benefit of anti-LAG3 has been extensively investigated in solid tumors including mRCC. A phase I trial evaluating the utility of a recombinant soluble LAG-3 fusion protein, IMP321, showed an acceptable toxicity profile and better clinical outcomes[98]. There are several ongoing clinical trials evaluating the efficacy of relatlimab, an anti-LAG3 antibody in combination with ICI in advanced solid tumors, including mRCC (NCT02996110, NCT05328908).

Cancer vaccines incorporating dendric cells, tumor-specific peptides, and oncolytic virus therapy have been investigated in various tumors. These have been found to induce antigen presentation and T-cell priming[99]. A clinical trial investigating a multipeptide vaccine with nivolumab has demonstrated immunologic activity with promising survival in high-risk melanoma[100]. Clinical trials investigating oncolytic virus therapy in advanced solid tumor malignancies are ongoing (NCT03206073, NCT05346484). A phase II trial investigating pexa-vec, an oncolytic vaccinia virus, has shown a 76% disease control rate including one CR in patients with mRCC[101]. Given the benefit of fecal microbiota transplant in germ-free mice to overcome resistance to PD-1 blockade, gut microbiota modulation is emerging as a strategy to improve the efficacy of ICI and reverse resistance[73]. As stress pathways and β-adrenergic signaling play a significant role in the enhancement of an immunosuppressive TME, thereby decreasing the clinical response of ICI, blocking of the adrenergic pathways would enhance the activity of ICI. This was evidenced in a phase I clinical trial in which the addition of propranolol, a β-blocker to pembrolizumab in metastatic melanoma, showed promising clinical activity[102].

CONCLUSION AND FUTURE DIRECTIONS

With the use of ICI, the survival of patients with mRCC has improved significantly. Although many patients have achieved durable clinical benefits, including CR, primary and acquired resistance to ICI is a significant challenge that remains to be addressed. This resistance may be due to various factors, such as alterations in the tumor microenvironment, activation of alternative signaling pathways, or overexpression of immune exhaustion markers. Thus, there is an unmet need to elucidate the mechanisms underlying immunotherapy resistance in metastatic renal cell carcinoma and to develop strategies to overcome this issue. Addressing this problem is crucial to improve the efficacy of immunotherapy and ultimately improve outcomes for patients with metastatic renal cell carcinoma.

DECLARATIONS

Authors' contributions

Manuscript writing, creating figures and editing: Roy AM
Conception, supervision and editing of the manuscript: George S

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Conflicts of interest

Roy AM declared that there are no conflicts of interest. George S is on the advisory boards of AVEO, Bayer, BMS, Corvus, Eisai, EMD Serono, Exelixis, Merck, Pfizer, QED Therapeutics, Sanofi/Genzyme, Seattle Genetics. George S receives institutional research funds from Agensys, Aravive, AVEO, Bayer, BMS, Calitehra, Corvus, Eisai, Exelixis, Gilead, Merck, Novartis, Pfizer, Seattle Genetics and Surface Oncology.

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Cancer Drug Resistance

Review

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A review of strategies to overcome immune resistance in the treatment of advanced prostate cancer

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Abstract

Immunotherapy has become integral in cancer therapeutics over the past two decades and is now part of standard-of-care treatment in multiple cancer types. While various biomarkers and pathway alterations such as dMMR, *CDK12,* and AR-V7 have been identified in advanced prostate cancer to predict immunotherapy responsiveness, the vast majority of prostate cancer remain intrinsically immune-resistant, as evidenced by low response rates to anti-PD(L)1 monotherapy. Since regulatory approval of the vaccine therapy sipuleucel-T in the biomarker-unselected population, there has not been much success with immunotherapy treatment in advanced prostate cancer. Researchers have looked at various strategies to overcome immune resistance, including the identification of more biomarkers and the combination of immunotherapy with existing effective prostate cancer treatments. On the horizon, novel drugs using bispecific T-cell engager (BiTE) and chimeric antigen receptors (CAR) technology are being explored and have shown promising early efficacy in this disease. Here we discuss the features of the tumour microenvironment that predispose to immune resistance and rational strategies to enhance antitumour responsiveness in advanced prostate cancer.

Keywords: Prostate cancer, immunotherapy, immune checkpoint inhibitor, immune resistance, tumour microenvironment

INTRODUCTION

Prostate Cancer has the second highest cancer incidence worldwide and is the 5th leading cause of cancer death in men[1]. The cornerstone treatment of locally-advanced and metastatic prostate cancer centres upon androgen deprivation therapy. Patients who experience disease progression while having castrate levels of testosterone are considered castration-resistant. In the advanced prostate cancer setting, additional treatment modalities include novel hormonal agents (NHAs), chemotherapy, radioligand therapy, poly(ADP)-ribose polymerase (PARP) inhibitors, and immunotherapy. Successive waves of clinical trials in the past decade have brought these treatment modalities forth from the castration-resistant setting into the hormone-sensitive setting, showing improved survival with early introduction of chemotherapy, NHAs, or combinations of these[2]. Despite these advances in prostate cancer treatment, the 5-year survival for metastatic prostate cancer patients in 2022 remains low at 32.3%[3].

Immunotherapy, in the form of sipuleucel-T, received FDA approval in 2010 for the treatment of patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC). In patients with deficient mismatch repair or microsatellite-high (dMMR/MSI-H) tumours, pembrolizumab and dostarlimab are FDA-approved options[4,5]. However, the prevalence of dMMR/MSI-H in prostate cancer is dismal at 1%, with *MSH2* being the most frequently implicated (other MMR genes being *MSH6, MLH1, PMS2*)[6]. Owing to an immunologically "cold" microenvironment in unselected acinar prostate adenocarcinoma, to date, no other immunotherapeutic agents have shown to be beneficial in the current treatment of advanced prostate cancer. In this review, we look at the current treatment paradigm, the role of immunotherapy, and existing and up-and-coming methods to overcome immune therapy resistance in prostate cancer.

IMMUNE REGULATION IN THE TUMOUR MICROENVIRONMENT (TME) OF PROSTATE CANCER

Immuno-oncology has changed the treatment paradigm of multiple tumour types, including melanoma, renal cell carcinoma, and lung carcinoma. The cancer-immunity cycle is depicted in Figure 1, explaining how the innate immune system fends off cancer cells and the various points at which therapeutic targets act. Despite successes in these typically immunogenic tumours, prostate cancer has traditionally been considered to have an immunologically "cold" tumour microenvironment (TME) characterized by T cell exclusion, low neoantigen load, and a highly immunosuppressive microenvironment comprising a high proportion of myeloid-derived suppressor cells (MDSCs)[7,8]. Factors that suggest a maladaptive immune response against tumour cells include lack of tumour-infiltrating lymphocytes (TILs), presence of M2-polarized tumour-associated macrophages (TAMs) and MDSCs, with evidence that increment in such cell populations within the TME is correlated with tumour progression[9]. MDSCs are immune cells that are activated in cancers and display potent immunosuppressive effects leading to prostate cancer resistance to anti-hormonal therapy[10]. Furthermore, CRPCs frequently exhibit *PTEN* loss, which is associated with increased MDSC infiltration[11] and may interact with the interferon-1 pathway required for innate immune activation[12]. Other immune-suppressive factors within the TME, such as soluble tumour necrosis factor (sTNF), interleukin-1 beta (IL-1β), TGF-β, and IL-10, promote chronic inflammation and increase myeloid cell differentiation into MDSCs[13,14].

Reduced immune stimulatory factors can also contribute to the immunologically cold TME in prostate cancer. CRPC patients have decreased peripheral natural killer (NK) cell pools, and this may be due to increased NK cell group 2 member D (NKG2D) serum receptor levels from the tumour[15]. This phenomenon is more pronounced with metastatic disease[9]. NK cells are lymphocytes that have roles in innate and adaptive immunity, whereas NKG2D is an activating cell surface receptor expressed on NK cells,

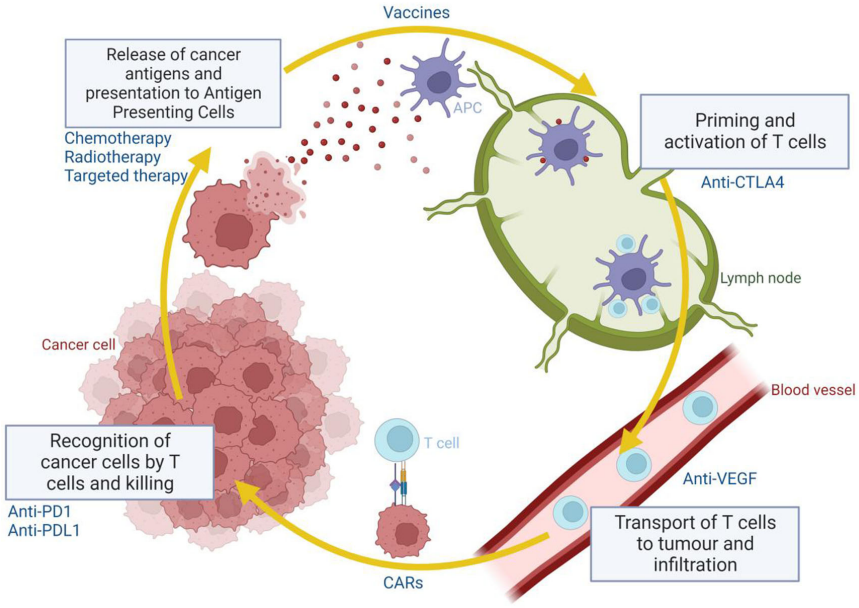

Figure 1. The cancer immunity cycle and where various classes of drugs act on.

NKT cells, and subsets of γδ T cells. Although initially thought to enhance immune responses against cancer, it appears that when NKG2D ligands are expressed chronically, this can instead lead to inhibition of immune cell function[16]. Low tumour mutational burden (TMB) in prostate cancer is associated with reduced neoantigen load recognised by the immune system[17]. These mechanisms enable immune evasion by cancer cells and directly impact the therapeutic response to anti-PD(L)1/anti-CTLA4 immune checkpoint inhibitors (ICIs)[18]. Figure 2 illustrates the interplay amongst the immune cells, cancer cells and vascular supply within the TME.

Potential biomarkers for ICI response include dMMR/MSI-H as mentioned above and tumours with DNA damage repair (DDR) pathway deficiencies. Tumours with DDR pathway deficiencies have increased mutational load as a result of decreased DNA repair capacity, leading to genomic instability[19]. Patients with somatic alterations in genes involved in DNA replication or repair have been shown to express higher neoantigen load, higher mutational burdens, higher levels of CD3+ and CD8+ TILs and higher PD-1/PD-L1 levels, all of which correlate with sustained ICI responses[20-24]. Despite this, dMMR and *CDK12*-altered prostate cancers have more aggressive biology[25,26]. A retrospective study of prostate cancer patients from the Royal Marsden Hospital showed that 8.1% of the patients had dMMR, which was correlated with decreased survival (median OS 4.1 years for dMMR *vs.* 8.5 years for proficient MMR)[26]. *CDK12* alterations were found in 6% of advanced prostate cancer in one study[25], and were typically linked to poor prognosis as well as insensitivity to PARP inhibitors[27]. However, these tumours have increased neoantigen load and tumoural lymphocyte infiltration, which may increase their response to ICIs[27].

ICI MONOTHERAPY IN THE UNSELECTED PROSTATE CANCER PATIENT

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a receptor found on the surface of T lymphocytes. When APCs activate T cells in response to the presence of foreign antigens, there is involvement of costimulatory molecules such as CD28 and B-7, which enhance the immune response. CTLA-4 acts as an immune checkpoint by binding to B-7, counteracting the costimulatory effect of CD28 and overall cause suppression of the immune response[28,29]. Cancer cells can downregulate the immune

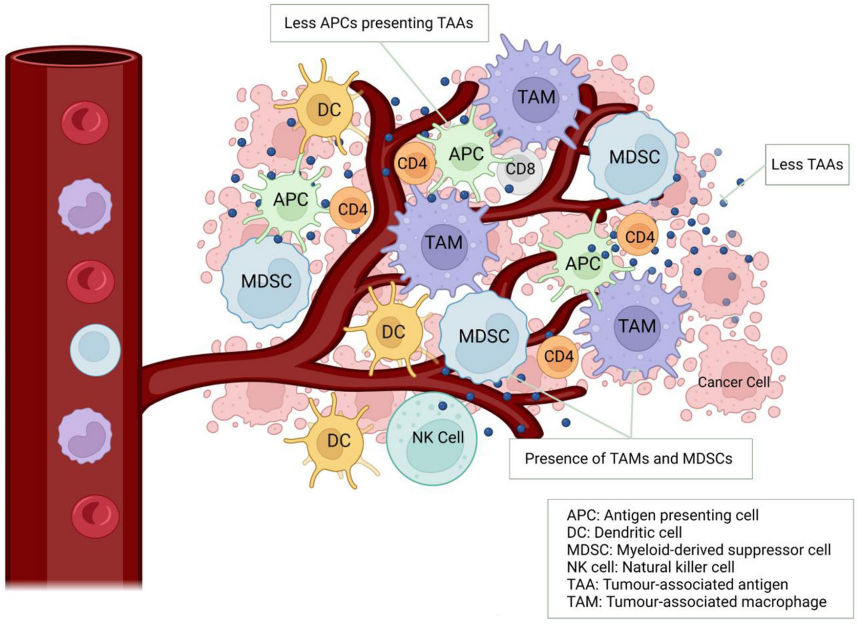

Figure 2. The immunologically "cold" tumour microenvironment in prostate cancer.

response by exploiting CTLA-4, and this forms the basis of targeting CTLA-4 with monoclonal antibodies such as ipilimumab. Inhibition of CTLA-4 activity causes activation and proliferation of cytotoxic T cells against tumour cells[30,31]. To date, two phase 3 trials have looked at the activity of ipilimumab in mCRPC patients. The first study, CA 184-043, recruited 799 mCRPC patients with at least one bone metastasis and have progressed on docetaxel chemotherapy. Patients were randomised to receive either one fraction of bone-directed radiation therapy followed by ipilimumab at 10 mg/kg or placebo. There was no overall survival benefit seen in this study (median OS 11.2 *vs.* 10 months, HR 0.85, 95% CI 0.72-1.00), but a progression-free survival (PFS) benefit (4.0 *vs.* 3.1 months, HR 0.70, 95% CI 0.61-0.82) was seen[32]. The second study by Beer *et al.* (2017) randomised 602 mCRPC patients who were chemotherapy-naive and had no visceral metastases to ipilimumab at 10 mg/kg *vs.* placebo. The study showed no overall survival benefit (median OS 28.7 *vs.* 29.7 months; HR 1.11, 95% CI 0.88-1.39), although a PFS benefit (median PFS 5.6 *vs.* 3.8 months; HR 0.67; 95% CI 0.55-0.81) was observed. Exploratory analyses further showed a higher prostate-specific antigen (PSA) response rate with ipilimumab (23%) than with placebo (8%)[33]. Taken together, the PFS and PSA response with ipilimumab suggests antitumour activity despite the lack of survival benefit.

PD-1 is a transmembrane glycoprotein found on the surfaces of activated cytotoxic T cells, B cells, dendritic cells, NK cells, and macrophages[34]. The binding of PD-1 to its ligands programmed death ligands 1 and 2 (PD-L1 and PD-L2) found on cancer cells delivers inhibitory signals for T-cell activation, suppressing an immune response[35,36]. Monoclonal antibodies targeting PD-1/PD-L1, such as nivolumab and pembrolizumab, have shown activity in multiple cancer types, leading to regulatory approval for their use[37,38]. Pembrolizumab was studied in the phase 1b KEYNOTE-028 and phase 2 KEYNOTE-199 trials as monotherapy in mCRPC, showing poor responses[39,40]. The objective response rate (ORR) was 5% in PD-L1 combined positive score (CPS) ≥ 1 patients in KEYNOTE-199, compared with 3% for patients with a negative PD-L1 expression[39]. Three phase 1 dose-escalation trials of nivolumab monotherapy in mCRPC patients likewise showed no objective response[41-43]. As mentioned, the paucity of PD-L1 expression in the TME in prostate cancer patients could account for this. Despite the glaringly low response rates for anti-PD(L)1/anti-CTLA4 monotherapies in unselected prostate cancer, the expression of immune

checkpoints has been reported to be dynamic, and various agents such as ipilimumab, sipuleucel-T and enzalutamide can increase T cell infiltration into the TME and modulate response to anti-PD(L)1 therapy[44]. This sets the stage for combination of various therapies with ICIs to improve immunotherapeutic response in prostate cancer.

ONGOING STRATEGIES TO OVERCOME IMMUNE RESISTANCE

Several strategies have been examined to modulate antitumour immunity in advanced prostate cancer.

PARP inhibitors and ICIs

PARP inhibitors are small molecules that prevent the repair of single-strand DNA breaks. Pathogenic DDR gene alterations are found in 23% of mCRPCs[45], with *BRCA2*, *ATM*, *CHEK2*, and *BRCA1* being the most frequently implicated genes[46]. The resulting homologous recombination deficiency (HRD) leads to sensitivity to PARP inhibition as a result of synthetic lethality[47]. Presently in mCRPC patients, the FDA has approved rucaparib for use in those with germline/somatic *BRCA* mutation and olaparib for those with germline/somatic homologous recombination repair (HRR) gene mutations. This is based on a high ORR of 50.8% seen with rucaparib use in the phase 2 TRITON2 study and improved radiologic PFS with olaparib use over enzalutamide/abiraterone in the phase 3 PROfound study[48,49]. The phase 3 TRITON3 study showed similar results[50]. Furthermore, efforts made in examining PARP inhibition in unselected patients have been successful as well, with the phase 3 PROpel trial showing improvement in radiologic PFS with combination abiraterone plus olaparib over abiraterone plus placebo as first-line treatment of mCRPC patients, overall suggesting an increasing role in PARP inhibition[51].

Increased micronuclei and cytosolic double-stranded DNA release after PARP inhibitor treatment as a result of PARP-DNA trapping and DNA damage leads to increased neoantigen formation, increased PD-L1 expression, increased intra-tumoural CD8 T cell infiltration and increased interferon production in the TME, forming the basis for ICI-PARP inhibitor combinations, and potentially expanding the benefit of PARP inhibitors beyond tumours harbouring alterations[52,53]. A phase 2 open-label clinical trial combining durvalumab with olaparib in men with mCRPC showed a response (radiographic or biochemical) in 9 out of 17 patients. Five of the 9 responders were found to have dysfunctional DDR genes based on genomic analysis and the presence of mutated DDR genes was associated with significantly higher 12-month PFS than those without (83.3% *vs*. 36.4%). Interestingly, patients with fewer peripheral MDSCs were more likely to respond[54]. This study showed early evidence of combining PARP inhibitors and ICIs, and other ongoing studies looking at similar combinations are listed in Table 1.

As mentioned, *CDK12*-altered prostate cancers typically carry poor prognosis and do not respond well to PARP inhibition, yet they present increased neoantigen load and lymphocytic infiltration, which may increase responsiveness to anti-PD1 therapy[25,27]. A retrospective study of 60 men with *CDK12*-altered advanced prostate cancer showed that of the 9 men who received PD-1 inhibitor therapy, 33% had a PSA response and the median PFS was 5.4 months[27,55]. Similarly, the ongoing phase 2 IMPACT trial has shown a 21.4% PSA response with ipilimumab-nivolumab combination in these patients[55].

Vaccines and ICIs

Anti-cancer vaccines can be classified into four groups: cell-based, viral-based, DNA/RNA-based, and peptide-based vaccines[56,57]. The goal of vaccine therapy is to stimulate the host's adaptive immune response against tumour-associated antigens (TAA). Prostate cancer is suitable for vaccine therapy because it has many TAAs such as PSA, prostate-specific membrane antigen (PSMA), prostate acid phosphatase (PAP), prostate stem cell antigen (PSCA), prostate cancer antigen 3 (PCA3), mucin-1, and six-transmembrane epithelial antigens of the prostate (STEAP)[58].

Table 1. Trials looking at ICI combinations in treatment of advanced prostate cancer

Trial number	Phase	Intervention arm(s)	Population	Outcome	Status
ICIs + PARP inhibitor					
NCT02484404	2	Durvalumab + Olaparib	mCRPC after progression with 1 NHA or Docetaxel	ORR, safety, DOR, PSA response	Completed
NCT04336943	2	Durvalumab + Olaparib	Recurrent prostate cancer with immunogenic signature	PSA response	Active, recruiting
NCT03834519	3	Pembrolizumab + Olaparib NHA (Abiraterone or Enzalutamide)	mCRPC after progression with 1 NHA and chemotherapy	OS, rPFS	Active, not recruiting
NCT02861573	1/2	Pembrolizumab + Olaparib Multiple cohorts	mCRPC	ORR, safety, PSA response	Active, recruiting
NCT05568550	2	Pembrolizumab + Olaparib + RT Pembrolizumab + RT	High-risk localised PC	PSA response	Not yet recruiting
NCT03338790	2	Nivolumab + Rucaparib Nivolumab + Docetaxel Nivolumab + Enzalutamide	mCRPC	ORR, PSA response	Active, not recruiting
NCT04592237	2	Maintenance Cetrelimab + Niraparib Maintenance Niraparib	Aggressive variant mPC given induction Cabazital + Carboplatin + Cetrelimab	PFS	Active, recruiting
ICIs + vaccines					
NCT03024216	1	Atezolizumab + Sipuleucel-T	mCRPC	Safety	Completed
NCT01832870	1	Ipilimumab + Sipuleucel-T	CRPC eligible to receive Sipuleucel-T in accordance to FDA-approved labeling	Antigen-specific T cell response, antibody response	Completed
NCT00113984	1	MDX-010 (anti-CTLA-4) + PROSTVAC-V/TRICOM (virus vaccine)	mCRPC after progression with anti-androgens and ≤ 1 chemotherapy	Safety	Completed
NCT02933255	1/2	Nivolumab + PROSTVAC-V/F	mCRPC Neoadjuvant therapy for localised PC planned for surgery	Safety, changes in T-cell infiltration	Active, recruiting
NCT03315871	2	M7824 (anti-PD-L1/TGFβ) + PROSTVAC + CV301 (virus vaccine)	CRPC	PSA response	Active, recruiting
NCT03532217	1	Ipilimumab + Nivolumab + PROSTVAC-V/F + Neoantigen DNA vaccine	mHSPC	DLT, safety, immune response	Completed
NCT03493945	1/2	M7824 (anti-PD-L1/TGFβ) + BN-Brachyury (virus vaccine)+ N-803 (IL-15 superagonist complex) + Epacadostat (IDO1 inhibitor)	CRPC	CBR	Active, recruiting
NCT02325557	1/2	Pembrolizumab + ADXS31-142 (bacteria vaccine)	mCRPC after progression on ≤ 3 systemic therapies	Safety	Unknown
NCT02499835	1/2	pTVG-HP + concurrent Pembrolizumab pTVG-HP + sequential Pembrolizumab	mCRPC	ORR, safety, PSA response, PFS	Active, not recruiting
NCT04090528	2	Pembrolizumab + pTVG-HP (DNA vaccine) + pTVG-AR HP (DNA vaccine) Pembrolizumab + pTVG-HP	mCRPC	PFS	Active, recruiting
NCT04382898	1/2	Cemiplimab + BNT112 BNT112 (RNA vaccine)	mCRPC after progression on 2-3 therapies including NHA and/or chemotherapy	DLT, ORR, Safety	Active, recruiting
ICIs + tyrosine kinase inhibitors					
NCT04446117	3	Atezolizumab + Cabozantinib + NHA (Abiraterone or Enzalutamide)	mCRPC after progression on 1 NHA	PFS, OS	Active, recruiting
NCT03170960	1/2	Atezolimab + Carbozantinib	mCRPC after progression on ≤ 1 NHA	DLT, ORR	Active, not recruiting
NCT04477512	1	Nivolumab + Cabozantinib + Abiraterone	mHSPC	DLT	Active, recruiting
NCT04159896	2	Nivolumab + ESK981 (Pan-VEGFR/TIE2 TKI)	mCRPC after progression on 1 NHA and 1 chemotherapy	Safety, PSA response	Unknown
Combination ICIs					
NCT04717154	2	Ipilimumab + Nivolumab	mCRPC with immunogenic signature	DCR	Active, recruiting
NCT03570619	2	Ipilimumab + Nivolumab	mCRPC with CDK12 aberration	ORR, PSA response	Active, not recruiting

NCT03061539	2	Ipilimumab + Nivolumab	mCRPC with immunogenic signature after progression on 1 systemic therapy	ORR, PSA response	Active, not recruiting
NCT02985957	2	Ipilimumab + Nivolumab Ipilimumab Cabazitaxel	mCRPC	ORR, rPFS	Active, not recruiting
NCT03333616	2	Ipilimumab + Nivolumab	Non-adenocarcinoma PC	ORR	Active, recruiting
NCT02788773	2	Durvalumab + Tremelimumab	mCRPC with prior exposure to 1 NHA	ORR	Active, not recruiting
ICIs + androgen receptor antagonist					
NCT03016312	3	Atezolizumab + Enzalutamide Enzalutamide	mCRPC with prior exposure to 1 NHA and 1 chemotherapy	OS	Completed
NCT02787005	2	Pembrolizumab + Enzalutamide	mCRPC progressing on Enzalutamide	ORR	Completed
NCT04191096	3	Pembrolizumab + Enzalutamide Enzalutamide	mHSPC	rPFS, OS	Active, not recruiting
NCT03834493	3	Pembrolizumab + Enzalutamide Enzalutamide	mCRPC, allows for prior Abiraterone exposure	rPFS, OS	Active, not recruiting
NCT02312557	2	Pembrolizumab + Enzalutamide	mCRPC after progression on Enzalutamide	PSA response	Active, not recruiting
NCT03338790	2	Nivolumab + Rucaparib Nivolumab + Docetaxel Nivolumab + Enzalutamide	mCRPC	ORR, PSA response	Active, not recruiting
NCT01688492	1/2	Ipilimumab + Abiraterone	mCRPC	Safety, PFS	Active, not recruiting
ICIs + chemotherapy					
NCT03338790	2	Nivolumab + Docetaxel	mCRPC	ORR, PSA response	Active, not recruiting
NCT04100018	3	Nivolumab + Docetaxel Nivolumab	mCRPC after progression on 1-2 NHAs	rPFS, OS	Active, recruiting
NCT03834506	3	Pembrolizumab + Docetaxel Docetaxel	mCRPC with prior exposure to 1 NHA	rPFS, OS	Active, not recruiting
NCT02861573	1/2	Pembrolizumab + Docetaxel Multiple cohorts	mCRPC	ORR, safety, PSA response	Active, recruiting
NCT03409458	1/2	Avelumab + PT-112 (Platinum + Pyrophosphate ligand)	mCRPC	Safety, PSA response	Active, not recruiting
NCT02601014	2	Nivolumab + Ipilimumab	AR-V7-expressing mCRPC	PSA response	Completed
NCT02788773	2	Durvalumab + Tremelimumab Durvalumab	mCRPC with prior exposure to 1 NHA	ORR	Active, not recruiting
ICIs + radiopharmaceuticals					
NCT02814669	1	Atezolizumab + Radium-223	mCRPC after progression on 1 NHA and 1 chemotherapy	ORR, safety	Completed
NCT04109729	1/2	Nivolumab + Radium-223	mCRPC with symptomatic bone metastases	Safety, ctDNA reduction	Active, recruiting
NCT03658447	1/2	Pembrolizumab + 177Lu-PSMA	mCRPC after progression on 1 NHA	Safety, PSA response	Completed

CBR: Clinical benefit rate; CRPC: castration-resistant prostate cancer; DCR: disease control rate; DLT: dose limiting toxicity; DOR: duration of response; ICIs: immune checkpoint inhibitors; mCRPC: metastatic castration-resistant prostate cancer; mPC: metastatic prostate cancer; NHA: novel hormonal agent; ORR: objective response rate; OS: overall survival; PARP: poly(ADP)-ribose polymerase; PC: prostate cancer; PFS: progression-free survival; PSA: prostate-specific antigen; rPFS: radiologic progression-free survival; RT: radionuclide therapy.

Sipuleucel-T is a therapeutic dendritic cell-based vaccine that has received FDA approval for use in the treatment of patients with asymptomatic or minimally symptomatic mCRPC, based on overall survival (OS)

benefit seen from the phase 3 IMPACT trial[59]. It is prepared from autologous peripheral blood mononuclear cells obtained by leukapheresis, and pulsed *ex vivo* with PAP2024, a unique fusion protein of granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP). GM-CSF fosters the maturation of dendritic cells and other APCs to present PAP to the patient's T cells, resulting in PAP-specific T-cell proliferation targeting the PAP-expressing prostate cancer cells for killing. Both humoural and cellular responses have been reported, with peripheral immune responses to PAP and measures of APC activation correlating with improvements in OS[60,61]. Despite success with the use of sipuleucel-T, other vaccines studied have not been as successful. G-VAX is another cell-based GM-CSF-secreting vaccine that utilises irradiated TAAs[62]. The TAAs are derived from two cell lines: one hormone-sensitive (LNCaP) and one hormone-resistant (PC3)[63]. Despite initially promising results in asymptomatic mCRPC, the phase 3 VITAL 1 and VITAL 2 trials in asymptomatic mCRPC and symptomatic mCRPC patients, respectively, failed to show the OS benefit of G-VAX plus docetaxel against docetaxel alone. Both studies were terminated early based on futility assessments. A viral-based vaccine, PROSTVAC, utilizes recombinant poxviruses that express PSA with immune-enhancing costimulatory molecules to stimulate immune response[64,65]. In addition to induced modified human PSA, they contain three costimulatory domains for T cells (B7.1, leukocyte function-associated antigen-3, and intercellular adhesion molecule-1), called TRICOM[66]. The phase 3 PROSPECT trial was unable to demonstrate the OS benefit of PROSTVAC against placebo control[67].

Given the increase in T cell infiltration and inflammation within TME with sipuleucel-T[60,61], it is therefore postulated that synergy might be observed with the combined use of vaccines and ICIs. Ipilimumab and PROSTVAC were combined in a phase 1 dose-escalation trial, showing evidence of improved clinical and immunologic outcomes. The median OS was 34.4 months[68], which appears to be numerically larger than PROSTVAC alone in its original study[67]. There was a PSA reduction in 54% of patients and a PSA decline of more than 50% was seen in 25% of patients. ADXS31-142 is a live, attenuated, bioengineered listeria-based vaccine targeting PSA. It is being studied as part of the KEYNOTE-046 trial, with current results showing a median OS of 33.7 months for patients treated with combination vaccine and pembrolizumab[69]. Other ongoing studies of vaccine therapy with ICIs are listed in Table 1.

Tyrosine kinase inhibitors and ICIs

Prostate cancers have dysregulated vasculature that promotes an immunosuppressive TME[7,8]. These include promoting a shift in TAMs toward M2-like immunosuppressive phenotype, reduced maturation of dendritic cells which results in reduced antigen presentation, and increased PD-L1 expression[70]. Vascular endothelial growth factor (VEGF) overexpression has been found to prevent the differentiation of monocytes into dendritic cells[71]. Meanwhile, an improvement in the regulation of local vascular in preclinical models was associated with the assimilation of TAMs with M1-like immune-stimulatory phenotype, increased CD4+ and CD8+ T-cell infiltration into the TME, and reduction of MDSCs[72-75]. These suggest that targeting angiogenesis in tumours can inhibit tumour-induced dysregulation of local vasculature and promote immunogenicity in the TME, forming the basis of combining antiangiogenesis agents with ICIs. Indeed, it has been shown in renal cell carcinoma that anti-VEGF therapy leads to a reduction in immune inhibitory stimuli such as regulatory T-cells and MDSCs[76,77]. Aside from VEGFR targeting, the TAM family of receptor tyrosine kinases comprising TYRO3, AXL and MER has been shown to promote immune suppression as well, making it an attractive target[78,79].

Cabozantinib is a multi-kinase inhibitor targeting MET, VEGFR-1, -2 and -3, AXL, RET, ROS1, TYRO3, MER, KIT, TRKB, FLT-3, and TIE-2[80]. Preclinical data suggests that it has an effect on the TME by reprogramming M2 TAMs to "pro-inflammatory" M1 macrophages, in addition to reducing MDSCs and T regulatory cells[81]. A dose-expansion cohort in the phase 1b COSMIC-021 trial evaluated the combination

of cabozantinib with atezolizumab (anti-PD1) in mCRPC patients who have had disease progression following treatment with novel hormonal agents such as abiraterone or enzalutamide. An ORR of 32% was observed in 132 patients treated with the combination, with a disease control rate (DCR) of more than 80%. This effect was consistent in patients with visceral disease as well[82]. Due to promising results from this study, this combination is now being evaluated in a phase 3 clinical trial for mCRPC patients. Other ongoing studies looking at combination anti-VEGF therapy with ICIs are listed in Table 1.

Combination ICIs

CheckMate-650 is a phase 2 study looking at various dosing combinations of nivolumab with ipilimumab in asymptomatic or minimally symptomatic mCRPC patients who have progressed on novel hormone therapy in two cohorts (chemotherapy-naive and chemotherapy-exposed). In the chemotherapy-naive cohort, nivolumab/ipilimumab achieved an ORR of 25% with a median radiological PFS of 5.5 months and a median OS of 19.0 months. In the chemotherapy-exposed cohort, the ORR was 10%, with a median radiological PFS of 3.8 months and a median OS of 15.2 months[83]. Exploratory analyses revealed that PD-L1 ≥ 1%, the presence of DDR or homologous recombination deficiency (if at least one gene in the relevant gene panel had a deleterious mutation/homozygous deletion) were associated with higher ORR[83]. In this study, 44 patients had quality-controlled whole-exome sequencing data, giving rise to a median TMB of 74.5 mutations/patient. Tumours harbouring TMB exceeding this median were associated with higher ORR, PSA response rate, radiologic PFS, and median OS[83].

Combination nivolumab and ipilimumab has been examined in AR-V7 expressing mCRPC patients as well. Androgen receptor splice variant 7 (AR-V7) expression is found in approximately 20% of mCRPC patients and is associated with alterations in a greater number of DDR genes, which could increase susceptibility to ICIs[84]. The STARVE-PC trial is a phase 2 non-randomised study that evaluated the activity of nivolumab and ipilimumab in 15 AR-V7 expression mCRPC patients, showing an ORR of 25%, PSA response rate of 13% and OS of 8.2 months[85]. Responses were more pronounced in six of the patients who were found to have mutations in DDR genes (three in *BRCA2*, two in *ATM,* and one in *ERCC4*)[86]. Lastly, an ongoing phase 2 randomised study is looking at mCRPC patients following progression on novel hormonal agents, randomising them to receive durvalumab or combination durvalumab plus ipilimumab. The ORR with combination ICI was 16% *vs.* 0% with durvalumab monotherapy in this study[87]. Other ongoing trials evaluating the efficacy of combination ICIs are listed in Table 1.

Androgen receptor antagonists and ICIs

How prostate cancer treatment impacts the immune response is variable. ADT enhances lymphopoiesis, which can mitigate immune tolerance to prostate cancer antigens[88]. On the other hand, androgen receptor antagonists have been shown to inhibit T cell responses[89].

ADT and anti-androgens can both target the AR signalling pathway and have been shown to result in an increase in the number of TILs, and a decrease in the number of regulatory T cells supporting an antitumour response to ADT[90,91]. Animal models confirm that while ADT induces pro-inflammatory conditions initially, the subsequent development of castration resistance and immune tolerance to prostate cancer antigens reduces this[92,93]. Therefore, the combination of AR-signalling blockade with ICIs, especially during its pro-inflammatory state, may be beneficial in the treatment of advanced prostate cancer.

The phase 2 IMbassador250 trial examined 759 advanced CRPC patients who had progressed on abiraterone and docetaxel, randomising them to receive combination enzalutamide and atezolizumab *vs.* enzalutamide alone. The study was closed prematurely due to futility (combination therapy *vs.* enzalutamide monotherapy, 15.2 *vs.* 16.6 months; HR 1.12, 95% CI 0.91-1.37). However, pre-planned

exploratory analyses showed a longer PFS with combination therapy in patients with high PD-L1 IC2/3, CD8 expression[94]. The study also performed an unbiased RNA sequencing-based analysis of immune-related gene expression that had previously correlated with mCRPC responses to immunotherapy[95], and found longer PFS with combination therapy in patients harbouring genes related to pre-existing immunity such as TAP-1, CXCL9, interferon signalling[94]. The multicohort phase 2 KEYNOTE-199 trial examined combination pembrolizumab with enzalutamide in mCRPC patients whose disease were refractory to enzalutamide. In the cohorts with measurable disease and bone-predominant disease (cohorts 4 and 5), the disease control rate was 51% and ORR was 12%. The duration of response was almost 6 months in 60% of responders[96]. This strategy is being evaluated further in an ongoing phase 3 trial [Table 1].

Systemic chemotherapy and ICIs

Chemotherapy may potentiate antitumour immunity by various mechanisms, including the release of TAAs and enhancing antigen presentation, stimulating the activity of cytotoxic T lymphocytes[97,98]. Importantly, chemotherapy may reduce immunosuppressive cell populations such as MDSCs and regulatory T cells, known to maintain prostate cancer immune evasion[99,100]. Preclinical studies have suggested that chemotherapy does improve antitumour immune responses, showing that the addition of taxanes can cause a shift in macrophage populations toward the M1-like (immune-activating) phenotype and reduce regulatory T cell and MDSC populations in mouse models[101,102]. The multicohort phase 2 trial CheckMate 9KD showed that combination nivolumab and docetaxel in 41 chemotherapy-naive mCRPC patients who have progressed on novel hormonal agents achieved an ORR of 36.8%, radiologic PFS of 8.2 months and PSA response of 46.3%[103]. KEYNOTE-365 is an ongoing multicohort phase 1b/2 study examining combination pembrolizumab and docetaxel in mCRPC patients, yielding an ORR of 18%, PSA response of 28%, radiologic PFS of 8.3 months, and OS of 20.4 months[104]. Ongoing phase 3 trials (CheckMate7DX and KEYNOTE-921) evaluating the superiority of combination chemotherapy with immunotherapy over chemotherapy alone will shed light in this area [Table 1].

Radiopharmaceuticals and ICIs

177Lu-PSMA-617 has gained regulatory approval for the treatment of mCRPC patients who have been treated with androgen receptor (AR) pathway inhibition and taxane chemotherapy, based on positive results on a phase 3 trial[105]. In murine models, targeted radionuclide therapy (TRT) may increase PD-L1 expression on T cells and the combination of TRT with ICIs leads to increased infiltration of CD8 T cells[106]. There is, hence, interest in combining radionuclide therapy with ICIs. Despite low clinical response (ORR 6.8%, PSA response 4.5%, radiologic PFS 3 months) seen on a phase 1b trial combining Atezolizumab and Radium-223 in mCRPC[107], the interim results of another phase 1b/2 PRINCE trial are relatively promising. In this study, 37 mCRPC patients who have progressed on a novel hormonal agent and docetaxel were treated with pembrolizumab and 177Lu-PSMA-617, yielding an ORR of 78%, PSA response of 73%, and 24-week radiologic PFS of 65%[108] [Table 1].

FUTURE DIRECTIONS AND CONCLUSIONS

Research is ongoing to identify more immunogenic targets and pair them with the multiple TAAs that prostate cancer expresses. Amongst these, cellular-based therapy is an area that deserves special mention. Adoptive cell therapy involves the engineering of patients' T lymphocytes to target specific viruses or tumours. The use of chimeric antigen receptors (CAR) allows for the creation of artificial T-cell receptors used in adoptive cell therapy[109]. A first-in-human phase 1 study of 13 CRPC patients tested PSMA-targeting CAR T cells armoured with a dominant-negative TGF-β receptor. TGF-β is an inhibitory factor found at high levels within the prostate TME. In this study, 4 patients had a ≥ 30% reduction in PSA and 1 patient had a > 98% reduction in PSA. Five patients experienced grade 2 or higher cytokine-release syndrome (CRS)[110]. Another CAR T therapy using P-PSMA-101, which targets PSMA, was evaluated in 10

heavily-pre-treated CRPC patients, yielding PSA decline in 7 patients, with 4 patients having > 50% reduction in PSA. CRS was seen in 60% of patients[111]. Other CAR T products targeting Epithelial cell adhesion molecule (EpCAM) and Natural Killer Group 2D (NKG2D) have shown activity in prostate cancer patients as well[112,113]. Other potential targets of interest with adoptive cell therapy include PSA, PAP, PSCA, and B7-H3[114], and Table 2 shows a list of ongoing clinical trials.

Bispecific T cell engager (BiTE) antibodies is another technology that has been developed to target TAAs such as PSMA in prostate cancer cells. Structurally, these are bispecific monoclonal antibodies that can crosslink TAAs with the coreceptors on T cells, generating an antitumour immune response. Pasotuxizumab is a bispecific monoclonal antibody that crosslinks CD3 and PSMA, and its efficacy has been studied in 16 mCRPC patients on a phase 1 trial, showing ≥ 50% decline in PSA in 3 patients, of which two were long-term responders treated for 14.0 and 19.4 months, respectively. 81% of the patients had adverse events of grade ≥ 3[115]. The efficacy of AMG 160, a BiTE product that binds CD3 on T cells and PSMA on cancer cells, was evaluated in mCRPC patients on a phase 1 trial. In the preliminary report, 27% of patients had confirmed PSA responses and 84% of patients experienced CRS (10% grade ≥ 3)[116]. The study also had a subset of patients who received AMG 160 with pembrolizumab, and such a combination will likely be examined in future studies as well. Other potential BITE targets including STEAP, CEACAM5, DLL3, HER2 are being studied[117,118], and a list of ongoing trials can be seen in Table 2. Figure 3 shows a schematic diagram of BiTE therapy.

On the horizon, relevant and novel targets to modulate antitumour immunity in prostate cancer may include the targeting of relevant immune-metabolic pathways, such as the adenosine receptor[119-121], or cytokine-directed efforts, such as IL-8 involved in the differentiation of TAM to M2 phenotype (promotes immune resistance and tumour metastasis)[122,123], IL-23 which is a cytokine secreted by MDSCs[124] and TGF-β which promotes tumour growth and immunosuppression in the TME[81]. Targeting cell signalling pathways such as the phosphoinositide 3-kinase/mammalian target of rapamycin (*PI3K/mTOR*) pathway has also been shown to downregulate immunosuppressive cells such as T regulatory cells and may have a role in improving ICI efficacy in prostate cancer[125,126]. For example, in prostate cancer mouse models, intermittent *PI3K* inhibition was able to alleviate *PTEN*-null cancer cell-intrinsic immunosuppressive activity and turn "cold" tumours into T cell-inflamed ones[127]. Novel immune checkpoints may be worth exploiting in prostate cancer. Increased expression of V domain Ig suppressor of T Cell activation (VISTA) was found to promote immune resistance following Ipilimumab treatment, which may serve as a new immunotherapeutic target in advanced prostate cancer[128].

There are presently limited biomarkers that can identify prostate cancer patients who may benefit from ICI therapy. It appears that combination strategies to promote immunogenicity within the "cold" TME of prostate cancer can increase the effect of ICIs. We recognise that the majority of the existing efforts are presently in the preclinical or early phase setting and may not be ready for use in the clinics yet. It would nevertheless be interesting to monitor this space for future developments.

Table 2. Trials looking at novel therapies in advanced prostate cancer

Trial number	Phase	Intervention arm(s)	Population	Outcome	Status
CAR T					
NCT04227275	1	CART-PSMA-TGFβRDN	mCRPC after progression on 2 NHAs	DLT, safety	Active, not recruiting
NCT03089203	1	CART-PSMA-TGFβRDN	mCRPC after progression on ≥ 1 systemic therapy	Safety	Active, recruiting
NCT04053062	1	LIGHT-PSMA-CART	mCRPC after progression on Abiraterone and chemotherapy	Safety	Suspended
NCT04249947	1	P-PSMA-101 CAR-T	mCRPC	ORR, DLT, safety	Active, not recruiting
NCT03873805	1	Anti-PSCA-CAR-4-1BB/TCRzeta-CD19t-expressing T-lymphocytes	PSCA+ mCRPC	DLT, safety	Active, recruiting
NCT02744287	1/2	BPX-601 (PSCA-specific CAR-T cells)	PSCA+ mCRPC	DLT, safety	Active, recruiting
NCT03013712	1/2	EpCAM-specific CAR T Cells	EpCAM+ mCRPC	Safety	Unknown
BiTE					
NCT04104607	1	CC-1 (PSMAxCD3)	mCRPC after progression on ≥ 3 systemic therapies	Safety	Active, recruiting
NCT03792841	1	Acapatamab (PSMAxCD3)	mCRPC after progression on 1 NHA and 1 chemotherapy	DLT, safety	Active, not recruiting
NCT01140373	1/2	HPN424 (PSMAxCD3)	mCRPC after progression on ≥ 2 systemic therapies	ORR, DLT	Active, not recruiting
NCT03972657	1/2	REGN5678 (PSMAxCD28) + Cemiplimab	mCRPC after progression on ≥ 2 systemic therapies	ORR, DLT, safety	Active, recruiting
NCT04221542	1	AMG 509 (STEAP1xCD3)	mCRPC after progression on 1 NHA and 1 chemotherapy	DLT, safety	Active, recruiting
NCT03406858	2	HER2Bi-armed activated T cells (HER2xCD3) + Pembrolizumab	mCRPC	PFS	Active, not recruiting

DLT: dose limiting toxicity; EpCAM: Epithelial cell adhesion molecule; mCRPC: metastatic castration-resistant prostate cancer; NHAs: novel hormonal agents; ORR: objective response rate; PFS: progression-free survival; PSCA: prostate stem cell antigen.

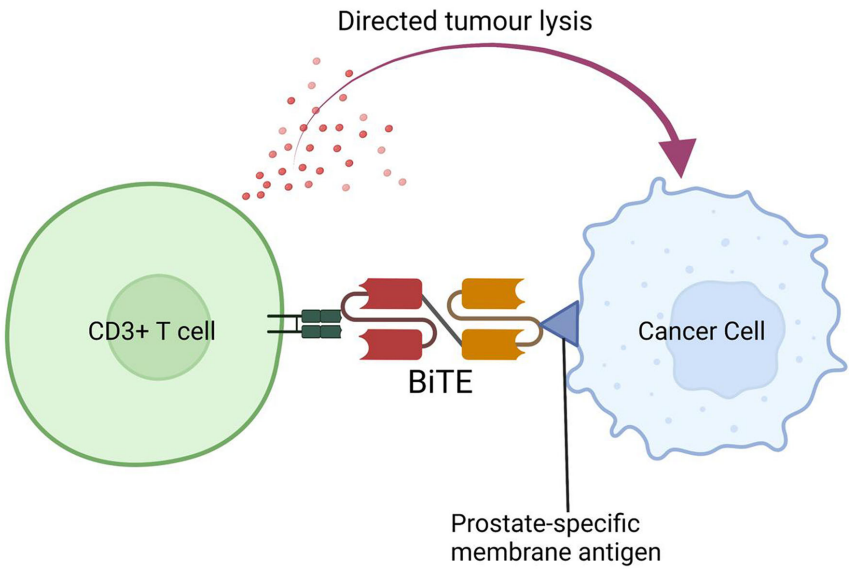

Figure 3. Bispecific T cell engager binding CD3 on T cell with PSMA on prostate cancer cell. BiTE: Bispecific T-cell engager; PSMA: prostate-specific membrane antigen.

DECLARATIONS

Authors' contributions

Conceptualisation: Sooi K, Wong A, Ngoi N
Methodology: Sooi K, Walsh R, Wong A, Ngoi N
Writing: Sooi K, Walsh R, Kumarakulasinghe N, Wong A, Ngoi N
Supervision: Kumarakulasinghe N, Wong A, Ngoi N
Visualisation: Sooi K

Availability of data and materials

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Unveiling T cell evasion mechanisms to immune checkpoint inhibitors in acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous and aggressive hematologic malignancy that is associated with a high relapse rate and poor prognosis. Despite advances in immunotherapies in solid tumors and other hematologic malignancies, AML has been particularly difficult to treat with immunotherapies, as their efficacy is limited by the ability of leukemic cells to evade T cell recognition. In this review, we discuss the common mechanisms of T cell evasion in AML: (1) increased expression of immune checkpoint molecules; (2) downregulation of antigen presentation molecules; (3) induction of T cell exhaustion; and (4) creation of an immunosuppressive environment through the increased frequency of regulatory T cells. We also review the clinical investigation of immune checkpoint inhibitors (ICIs) in AML. We discuss the limitations of ICIs, particularly in the context of T cell evasion mechanisms in AML, and we describe emerging strategies to overcome T cell evasion, including combination therapies. Finally, we provide an outlook on the future directions of immunotherapy research in AML, highlighting the need for a more comprehensive understanding of the complex interplay between AML cells and the immune system.

Keywords: Acute myeloid leukemia, T cells, immune checkpoint, immune evasion

INTRODUCTION

Acute myeloid leukemia (AML) is a devastating blood cancer and is the most common form of acute

leukemia in adults. Long-term outcomes for AML have not significantly improved over the past few decades, with a suboptimal 5-year overall survival rate of 30% for AML patients ages 20 and older and less than 10% for AML patients ages 65 and older[1]. The current standard of care approaches for AML, including induction chemotherapy, combinations of venetoclax with hypomethylating agents, and stem cell transplantation, still yield high relapse rates with significant toxicities. Therefore, new less toxic therapeutic approaches need to be developed to improve survival and prevent relapse in this disease.

Hematopoiesis is the process through which all mature blood cell lineages are generated from hematopoietic stem cells (HSCs), which have the capacity to both self-renew and differentiate. Without proper regulation of their cell-intrinsic and cell-extrinsic cues (primarily signaling pathways, transcription factors, and epigenetic regulators), HSCs and downstream progenitors can acquire unlimited self-renewal potential at the expense of differentiation, as well as increased proliferation and survival, leading to AML development[2-5]. AML blasts develop from aberrant HSCs or progenitors - termed the leukemic stem cell (LSC). LSCs are undifferentiated blood cells that have pathologic self-renewal properties and lead to abnormal blood production. Phenotypically, LSCs share some of the same cell surface markers as HSCs, but unique LSC and pre-LSC gene expression signatures have been identified by high throughput sequencing[6-9]. Like HSCs, LSCs are primarily quiescent and are therefore resistant to chemotherapy and other therapies that target actively cycling cell populations[3,10]. Yet, the standard induction "7 + 3" chemotherapy regimen remains the preferred up-front treatment strategy for AML patients who are fit enough to tolerate intensive induction therapy, which, in addition to sparing LSCs, results in various toxicities, such as pancytopenia and infection[11,12]. This has led to enhanced research efforts to identify novel therapies that target the LSC population while sparing healthy HSCs to improve AML patient outcomes.

However, in addition to the cell-autonomous mechanisms AML cells have employed to persist despite the cytotoxic effects of chemotherapy, AML cells have developed additional ways to persist despite treatment, including resistance mechanisms to targeted therapies and immune evasion. Notably, AML cells employ several mechanisms, such as reliance on immune cells, to establish an immunosuppressive environment to ensure their survival. This is accomplished through the reduction of cytotoxic and effector T and NK cells, increased T cell exhaustion, and recruitment of immunosuppressive populations such as regulatory T cells, myeloid-derived suppressor cells (MDSCs), and M2 macrophages[13]. Importantly, it has been reported that the number of effector and cytotoxic T cells, termed tumor-infiltrating lymphocytes (TILs), present in the bone marrow can be a prognostic marker for overall survival and leukemia-free survival[14]. In addition, increased numbers and function of regulatory T cells in both the peripheral blood and bone marrow of AML patients have been reported, with bone marrow-resident regulatory T cells exhibiting more immunosuppressive effects on CD4+ effector T cell proliferation[15]. A lower frequency of regulatory T cells was found to correlate with complete remission rates in AML patients, while a higher frequency was observed in patients who relapsed[15].

Despite advances in immunotherapies in solid tumors and some lymphoid malignancies, AML has been particularly difficult to treat with immunotherapies, primarily due to poor T cell recruitment to the bone marrow and because LSCs are immune privileged. Because even with the current therapeutic options, AML remains a lethal disease with a suboptimal long-term survival rate, it is imperative to identify and exploit the mechanisms by which AML cells evade immune detection to unleash the potential benefits of immunotherapy in AML treatment. This review summarizes the roles of T cells in the immune response, and highlights the challenges that AML cells pose to the efficacy of ICIs by evading T cell detection.

T CELLS FRONT THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune system plays an essential role in eliminating a variety of threats to our bodies, including cancer and infection. Key players in the adaptive immune response are B lymphocytes (B-cells) and T lymphocytes (T cells). They are distinguishable from cell types that primarily function in the innate immune response because they have antigen-specific receptors - B-cell receptor (BCR) and T cell receptor (TCR), respectively[16]. T cells can differentiate into three different cell types: effector T cells, cytotoxic T cells, and regulatory T cells. Effector T cells, also known as "helper T cells", which express the cell-surface protein CD4, function through cytokine signaling, such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), which stimulate other immune cells[16]. Cytotoxic T cells, which express the cell-surface protein CD8, program invading cells to undergo apoptosis via the secretion of granzyme B, perforin, and IFNγ[16]. Unlike effector and cytotoxic T cells, regulatory T cells function to suppress immune cells to mitigate any possible damage from a prolonged immune response, and to prevent auto-immunity[16]. They can be identified through flow cytometry by the expression of CD4, CD25, and FoxP3[16].

In order to activate a T cell-mediated immune response, two different signals are required. The first signal occurs when the disease-causing cell presents an antigen, or host-derived protein molecule, to a T cell [Figure 1]. Specifically, short peptide fragments of an antigen are presented on the surface of host cells, termed antigen-presenting cells (APCs), by major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules, MHC class I and MHC class II. Notably, CD8+ T cells selectively recognize MHC class I molecules, while CD4+ T cells selectively recognize MHC class II molecules. MHC class II molecules are often expressed on dendritic cells and macrophages, which engulf the antigen and process it for presentation. MHC class II molecules can also be present on the surface of foreign APCs. The Class II transactivator (CIITA) is a master regulator of MHC gene expression[17]. CIITA responds to IFNγ activation, where it then acts as a transcriptional activator to turn on MHC gene expression[17].

The second signal required for T cell activation is termed the co-stimulatory signal, and co-stimulation is thought to occur through the interaction between the CD28 molecule on T-lymphocytes and either CD80 (B7.1) or CD86 (B7.2) molecules on the APC[18] [Figure 1]. The discovery of CD28 and its essential role in T cell activation has led to further discovery of other cell-surface molecules that regulate T cell activity. Interestingly, the discovery of cytotoxic T lymphocyte antigen 4 (CTLA4) on T cells identified another binding partner of B7-1. However, CTLA4 expression is induced following T cell activation, where it can out-compete CD28 binding to B7.1 to dampen the T cell response[18].

This has led to the discovery and categorization of other cell-surface molecules that positively (referred to as co-stimulatory receptors) and negatively (co-inhibitory receptors) modulate T cell activity. Other co-inhibitory receptors on T cells include programmed cell death protein 1 (PD1), which binds to its ligands programmed death-ligand 1 (PD-L1, also known as B7-H1) or programmed death-ligand 2 (PD-L2, also known as B7-H2) on APCs; T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), and lymphocyte activation gene-3 (LAG3)[19,20]. TIM3 binds to various ligands (including Galectin-9, Ceacam-1, and HMGB-1), while LAG3 binds to MHC class II molecules with higher affinity than the CD4+ TCR[19,20]. Other co-inhibitory ligands on APCs include B7-H3, B7-H4, and B7-H5[21].

MECHANISMS OF IMMUNE EVASION IN AML

There are currently several different immunotherapy strategies being investigated in hematologic malignancies, including in AML[11,21]. Immune checkpoint inhibitors, such as antibodies targeting CTLA4 and PD1, have been approved for the treatment of some types of lymphoma and some solid tumors, including melanoma, lung cancer, kidney cancer, head and neck cancer, bladder cancer, and colorectal

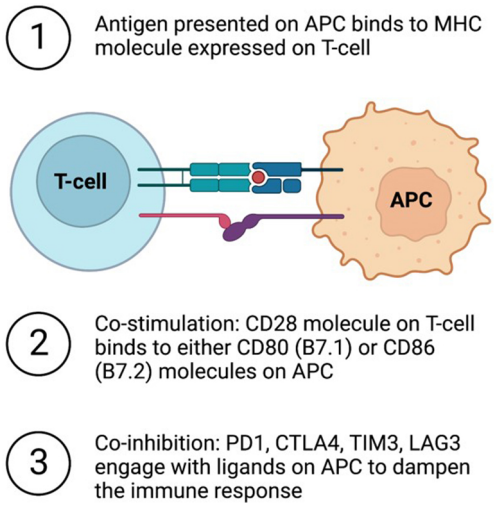

Figure 1. T cell-mediated immune response. Overview of the steps required for full T cell activation. Figure created with Biorender.com. APC: Antigen-presenting cell; CTLA4: cytotoxic T lymphocyte antigen 4; LAG3: lymphocyte activation gene-3; MHC: major histocompatibility complex; PD1: programmed cell death protein 1; TIM3: T cell immunoglobulin and mucin domain-containing protein 3.

cancer[22]. However, in AML, the use of immune checkpoint inhibitors has been more challenging, and there are no FDA approvals of this class of agents in AML to date. This is in part due to the various cell-autonomous and cell non-autonomous mechanisms that leukemic cells employ to reprogram themselves and the bone marrow microenvironment to render them immune privileged [Figure 2]. Additionally, ICIs are often most effective in cancers with a high mutation burden (i.e., melanoma, lung cancer), which is often not as high in AML[23]. For example, many AML patients have a defined blast population with 1-2 driver mutations and/or cytogenetic alterations, with sub-clones that may not arise until disease progression or relapse[24].

Increased immune checkpoint expression

Immune checkpoints are known to be a key mechanism that mediates T cell immunosuppression in AML. Interesting work using PD1 knockout mice delineated the importance of this axis in regulatory T cells and CD8+ cytotoxic T cells. Specifically, AML development was impeded when AML cells were injected into PD1 knockout mice[25]. This was dependent on the ability of regulatory T cells to suppress CD8+ T cells via enhanced PD1 expression on T cells and PD-L1 expression on APCs[25]. Interestingly, treating mice that developed AML with IL-2 linked to diphtheria toxin (IL-2DT), followed by anti-PDL1 monoclonal antibody treatment, markedly reduced the AML tumor burden[25]. Therefore, this work suggests that strategies to deplete regulatory T cells and inhibit the PD1/PD-L1 interaction could be effective in overcoming the AML-privileged microenvironment.

T cell exhaustion is also a mechanism for immune evasion and is often phenotypically characterized by the expression of the immune checkpoint TIM3. In one study evaluating the role of exhausted T cells in AML relapse following transplantation, the frequency of PD1-high TIM3-positive T cells was significantly correlated with AML relapse[26]. These T cells were confirmed to be exhausted, as they exhibited reduced production of IL-2, TNFα, and IFNγ[26]. The impact of this study was clinically significant, as the expression of exhaustion markers on T cells could be detected before the diagnosis of relapse[26]. These results were echoed in a subsequent study that characterized the exhausted T cell population following AML relapse post-transplantation, which, despite displaying specific leukemic blast recognition (determined by CDR3

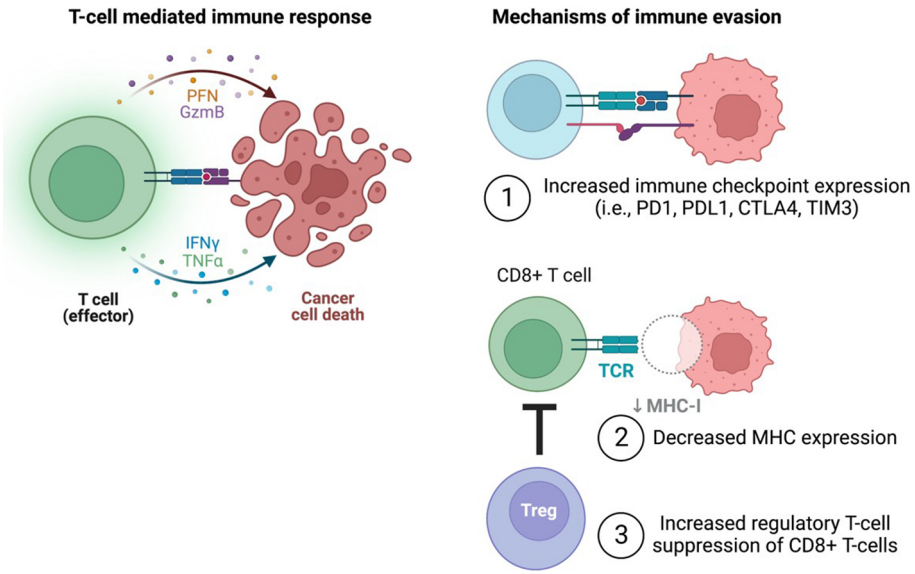

Figure 2. Mechanisms of T cell evasion in AML. T cells engage with and kill cancer cells through the presentation of MHC molecules and subsequent T cell co-stimulation (left). Mechanisms to evade T cell detection employed by AML cells include (1) increased expression of co-inhibitory immune checkpoints; (2) decreased MHC expression; and (3) suppression of cytotoxic CD8+ T cell function through increased regulatory T cells (right). Figure created with Biorender.com. AML: Acute myeloid leukemia; CTLA4: cytotoxic T lymphocyte antigen 4; IFNγ: interferon gamma; MHC: major histocompatibility complex; PD1: programmed cell death protein 1; PFN: perforin; TCR: T cell receptor; TIM3: T cell immunoglobulin and mucin domain-containing protein 3; TNFα: tumor necrosis factor alpha.

sequencing of TCR-α and TCR-β chains), had impaired effector T cell function[27]. As the prognosis for patients who relapse after transplantation is poor, early detection of T cell exhaustion markers could be a useful predictive tool[26,27].

Modulation of checkpoint expression on AML cells themselves is another key driver of immune evasion. For example, increased PD-L1, PD-L2, and CTLA4 expression on AML cells has been shown to correlate with poor overall survival[28,29]. PD-L1 expression was found to be elevated in AML patient blasts, both at diagnosis and at relapse[30]. Furthermore, CTLA4 was previously discovered to not be restricted to the lymphoid lineage, as AML cells from both diagnostic and relapsed patients, but not healthy CD34+ cells, were found to express CTLA4[31,32]. Therefore, the upregulation of inhibitory immune checkpoints on AML cells is another potential mechanism for immune evasion in AML.

Downregulation of MHC expression

Dampening of MHC expression on AML cells is also an important mechanism of immune evasion. Specifically, RNA sequencing analysis of paired AML samples collected at diagnosis and at relapse post-transplantation identified altered expression of immune-related genes, including decreased expression of CIITA, the master regulator of MHC-II expression, and of MHC-II molecules at relapse[33]. *Ex vivo* treatment of AML blasts isolated from relapse patients with IFNγ was able to restore MHC-II expression[33]. The clinical significance of this is revealed by the differences in CD4+ effector T cell activation, as measured by IFNγ production, following co-culture of either diagnostic or post-transplantation relapsed AML samples with CD4+ T cells, as CD4+ T cell activity was diminished in post-transplantation relapse co-culture assays[33].

Accordingly, in a recent transcriptome analysis of AML cells from patients who relapsed following transplantation, a transcription factor complex consisting of IRF8, MYB, MEF2C, and MEIS1 was found to regulate MHC expression in AML, and combinatorial changes in their expression are essential for reduced MHC expression at relapse[34]. Interestingly, the authors found a small cell population with silenced MHC expression at leukemia diagnosis, and concluded that, similar to resistant LSC populations, this population may be selected after transplantation and can contribute to relapse[34]. Overall, these mechanisms are plausible explanations for why the treatment of patients who relapse post-transplantation is particularly challenging. Identifying ways to overcome decreased MHC expression following transplantation is underway. For example, a recent study using AML xenograft mouse models reported that MDM2 inhibition can increase MHC-II production, and CD8+ T cells isolated from MDM2 inhibitor-treated primary AML mice can eradicate disease in secondary recipients[35].

The role of regulatory T cells in the immunosuppressive microenvironment

The increased number and activity of regulatory T cells (Tregs) in the leukemic bone marrow renders the bone marrow an immunosuppressive microenvironment due to their effects on effector and cytotoxic T cell populations. Recent insights have identified mechanisms for increased Treg function in the AML microenvironment, such as via increased expression and production of IFNγ by AML cells, leading to upregulation of genes that promote differentiation into Tregs[36]. Recognizing the correlation between increased Treg numbers and poor AML outcomes, one group investigated the effects of Treg ablation on leukemogenesis[37]. Using Foxp3-DTR to ablate Tregs in mice, they observed prolonged survival in MLL-AF9-induced AML mouse models and increased CD8+ T cell activity[37]. As Treg ablation is likely not easily transferrable to the clinic, they also identified additional ways to impede Treg accumulation in the leukemic microenvironment in mice, including CCL3-CCR1/CCR5 and CXCL12-CXCR4 blockade[37]. Importantly, as increased regulatory T cell populations are also a predictor of AML relapse following transplantation[27], it is critical to exploit mechanisms that decrease Treg numbers and function.

NK cell-mediated immune evasion mechanisms

AML cells can also evade detection by NK cells, which are canonically activated by the recognition of stress-induced ligands on foreign cells[38]. Similar to their evasion of T cells, AML cells can also evade NK-cell recognition and elimination through multiple mechanisms, including (1) the reduced expression of stress-induced ligands on AML cells; (2) increased expression of inhibitory receptors on NK cells to suppress NK cell function; (3) the induction of the immunosuppressive environment to limit NK cell numbers and function; and (4) activation of anti-apoptotic pathways to resist NK-cell induced cell death[39,40]. These NK-cell evasion mechanisms, as well as strategies to target them, have been extensively reviewed elsewhere[39,41-44]. For example, it was shown that epigenetic mechanisms mediate the silencing of NKG2D ligands in AML, and that treatment with hypomethylating agents can increase their expression and subsequent NK-cell recognition[45]. Furthermore, pivotal work demonstrated that LSCs are immune privileged through their lack of expression of NKG2D ligand, which is essential for NK-cell detection and subsequent clearance[46]. As NK2GD remains a hot target for immunotherapy in AML[47-49], it is important to appreciate that other mechanisms may be required to eliminate the LSC population.

Another mechanism that can mediate NK-cell evasion is CD48 silencing[50-52]. It was demonstrated that high CD48 expression on AML cells is correlated with a favorable prognosis. However, in a subset of AML patients, CD48 expression can be suppressed through enhanced methylation[52]. Therefore, treatment with hypomethylating agents may be able to increase CD48 expression to increase NK-cell killing[53]. Overall, understanding NK-cell evasion mechanisms is critical to overcoming immunotherapy resistance and identifying targets for immunotherapy.

CLINICAL INVESTIGATION OF IMMUNE CHECKPOINT INHIBITORS IN AML

Several strategies that incorporate checkpoint inhibitors have been tested in AML in clinical trials, and several more clinical trials are underway [Table 1]. In a phase 1/1b clinical trial of ipilimumab (anti-CTLA4) in patients with hematologic malignancies that relapsed after allogeneic stem cell transplantation, analysis of the AML subset (12/28 patients) showed that 5/12 patients achieved complete remission following treatment, and this was accompanied by a reduction in the frequency of circulating Tregs compared to non-responders[54].

Furthermore, in a Phase II study investigating the combination of high-dose cytarabine and pembrolizumab (anti-PD1) in relapsed/refractory AML patients, 14 out of 37 patients achieved complete remission (CR). Interestingly, of the patients that achieved a CR, TCR signaling identified a trend towards increased TCR diversity in these patients, as well as decreased regulatory T cell and increased CD8+ T cell frequencies[55]. Of note, RNA-seq analysis of AML blasts from these patients revealed that increased MHC expression was significantly upregulated at baseline in patients who achieved CR compared to non-responders[55].

Interestingly, recent data suggests that PD1 signaling may be implicated in the poor response to hypomethylating agents (HMAs), including azacitidine and decitabine, as patients who are resistant to HMAs show higher expression of PD-L1, PD-L2, and CTLA4[56-58]. On the other hand, preclinical findings from single-agent immune checkpoint inhibitor trials in AML have demonstrated limited efficacy. This has prompted the investigation of checkpoint inhibitors in combination with HMAs[57]. In a phase 1b trial investigating the combination of ipilimumab with decitabine in relapsed/refractory AML, patients who were transplant naïve (N = 23) observed a higher response rate than those who relapsed following stem cell transplantation (N = 20) (CR + CRi + mCR 52% *vs.* 20%, P = 0.034; median overall survival 16.2 months *vs.* 8.6 months)[59]. Not surprisingly, when performing integrative transcriptome-based analysis of bone marrow infiltrating cells from participating patients, a high baseline ratio of T cells to AML cells was associated with higher response rates[60]. The authors speculated that the inadequate clearance of the immature LSC population triggered relapse in patients following stem cell transplantation, but also noted that ipilimumab exposure resulted in increased memory T cell bone marrow infiltration and high expression of CTLA4 and FOXP3, suggesting that the efficacy of ipilimumab and decitabine may be impacted by these immune evasion mechanisms employed by LSCs[60]. The results of the ipilimumab and decitabine combination studies also highlight the limitations of ICIs in AML. A comparison of the memory and exhaustion gene scores associated with CD8+ T cells from AML bone marrow with those from CD8+ TILs isolated from solid tumors, in which ipilimumab demonstrates high clinical activity, revealed higher exhaustion profiles and checkpoint expression in solid tumor-derived T cells[60].

In two ongoing trials testing the combination of pembrolizumab and decitabine in relapsed/refractory AML, interim results showed a tolerable safety profile with promising efficacy data[56,61]. Furthermore, through the generation of RNA expression datasets from patients who were treated with conventional cytotoxic chemotherapy or with pembrolizumab and azacitidine in relapsed/refractory AML, Rutella *et al.* revealed a newly defined CD8+ T cell senescent gene population with a distinct gene expression signature[62]. These cells were impaired in their ability to kill AML blasts isolated from the same patient sample, and their frequency negatively correlated with overall survival[62]. However, there is still promise for the combination of PD1 blockade and HMA, as results from the Phase II trial investigating nivolumab and azacitidine in relapsed/refractory AML yielded a 33% overall response rate, with a higher response rate in HMA naïve *vs.* HMA pre-treated patients (58% *vs.* 22%)[63]. Based on these clinical trials, the possible predictors of response to immune checkpoint inhibitors are summarized in Table 2. Overall, given these data, the field is anxiously awaiting the results of additional clinical trials currently that are investigating

Table 1. Overview of ongoing clinical trials of immune checkpoint inhibitors in AML

Target	Agent	Regimen	Population	Phase	NCT identifier	Primary endpoints
PD-1	Pembrolizumab	IC ± Pembrolizumab	ND AML	2	NCT04214249 (BLAST MRD AML-1)	MRD-CR
		VEN + AZA ± Pembrolizumab	ND AML	2	NCT04284787 (BLAST MRD AML-2)	MRD-CR
		HiDAC followed by Pembrolizumab	R/R AML	2	NCT02768792	CR
		Decitabine + Pembrolizumab ± VEN	ND or R/R AML	1	NCT03969446	Incidence of AE, MTD, CR
	Nivolumab	Nivolumab	AML patients in remission after IC	2	NCT02275533 (REMAIN TRIAL)	PFS
		Nivolumab	AML patients in remission after IC	2	NCT02532231	Recurrence-free survival
		AZA + Nivolumab ± Ipilimumab	ND or R/R AML	2	NCT02397720	MTD, ORR
		Decitabine + VEN + Pembrolizumab	ND *TP53*-mutant AML	1	NCT04277442	Incidence of AE, CR
		Nivolumab ± Ipilimumab	AML patients post-HSCT	1	NCT03600155	Optimal dose
CTLA-4	Ipilimumab	Decitabine + Ipilimumab	R/R AML	1	NCT02890329	MTD
		Ipilimumab + CD25hi Treg-depleted DLI	R/R AML post-HSCT	1	NCT03912064	MTD

AE: Adverse event; AML: acute myeloid leukemia; AZA: Azacitidine; CR: complete remission; DLI: donor lymphocyte infusion; HSCT: hematopoietic stem cell transplant; HiDAC: high dose cytarabine; IC: intensive chemotherapy; MRD-CR: minimal residual disease negative complete remission; MTD: maximum-tolerated dose; ND: newly diagnosed; ORR: overall response rate; PFS: progression-free survival; R/R: relapsed/refractory; VEN: Venetoclax. Source: clinicaltrials.gov.

immune checkpoint inhibitors in AML.

Further investigation into the molecular mechanisms that both AML cells and T cells employ to evade immune detection may help to identify novel combination strategies for ICIs in AML. For example, altered signaling and expression of cellular proteins due to genetic alterations are hallmarks of AML cells. With both approved and investigational therapies available to target oncogenes (e.g., FLT3, IDH1/2, NPM1c/Menin inhibitors) responsible for regulating the expression and/or post-translational modifications (e.g., methylation, acetylation, glycosylation, ubiquitination) of proteins in AML cells, it is critical to determine if targetable driver mutations are important for the increased expression of immune checkpoints in AML cells.

Alternatively, further investigation into the mechanisms that T cells employ to increase checkpoint expression or to increase Treg function is warranted to improve ICI outcomes in AML. For example, a recent study analyzing the transcriptome of CD8 T cells from the bone marrow of AML patients demonstrated the downregulation of genes responsible for T cell activation, differentiation, and function (e.g., NF-KB, FOXO, cytokine/chemokine signaling)[64]. With several of these genes being involved in epigenetic regulation, the authors postulate that epigenetic changes to T cells may impair TCR activation and overall T cell function[64]. However, additional studies are necessary.

Lastly, additional studies are underway to identify mechanisms that increase the frequency of Tregs, with some insights regarding tumor necrosis factor receptor-2 (TNFR2) and the TNFα pathway playing an important role in increasing the frequency of Tregs in AML patient samples[65], in addition to increased expression of IFNγ via *IDO1* overexpression in mesenchymal stem cells[36]. Importantly, the mechanisms

Table 2. Possible predictors of response to immune checkpoint inhibitors in AML or MDS

Immune checkpoint inhibitor	Clinical setting	Possible predictors of response	Response assessment	Ref
Ipilimumab	Post-HSCT	-Baseline donor T cell chimerism of > 99% -Lower frequency of CD4+ Tregs -Increase in plasma CXCL2, CXLC5, CXCL6, IL1R, ANGPT-1 and -2, VEGF	CR or stable disease	[54]
Pembrolizumab	R/R AML, post-HiDAC	-Trend towards higher TCR diversity at baseline -Higher frequency of senescent T cells in BM -Higher frequency of terminally differentiated effector T cells in PB -Increased frequency of CD8+ T cells expressing CD28, PD-1, and TIGIT in BM -Presence of pre-treatment CD8+ T cells co-expressing TCF-1 and PD-1 -Transcriptional upregulation of PI3K/AKT/MTOR signaling pathway in BM blasts	CR	[55]
Ipilimumab	In combination with decitabine in AML or MDS before and after HSCT	-No clear predictors of response	Leukemic cell burden, frequency of infiltrating lymphocytes	[59]
Ipilimumab	In combination with decitabine in AML or MDS before and after HSCT	-Lower VAF of recurrent AML/MDS-associated mutations -Higher T cell to AML ratio -Increased T cell to myeloid ratio -Donor-derived myeloid cells present at higher % in responders -Higher circulating expression of CCL17, CXCL1, CXCL5, EGF, LAMP3, and PDGF subunit B	CR/CRi	[60]
Pembrolizumab	In combination with decitabine in R/R AML	-Trend towards increased CD3+ infiltrates in BM during treatment -No association of TCRb sample clonality with response	CR	[61]
Pembrolizumab	In combination with azacitidine in newly diagnosed AML *vs.* cytotoxic chemotherapy	-Increased proportion of CD3+CD8+CD57+KLRG1+ senescent T cells in baseline BM associated with worse OS -Increased proportion of senescent T cells in BM post-treatment associated with worse OS -High IED signature score associated with worse OS	OS	[62]
Nivolumab	In combination with azacytidine in R/R AML	-Trend towards association with improved response: no prior HMA, presence of ASXL1 mutation -Higher frequency of pre-treatment BM % CD3+ T cells in responders -Trend towards higher frequency of CD4+ T effector cells and CD8+ T cells in pre-treatment BM in responders	ORR	[63]

AML: Acute myeloid leukemia; BM: bone marrow; CR: complete remission; CRi: incomplete remission; HiDAC: high-dose cytarabine; HMA: hypomethylating agent; HSCT: hematopoietic stem cell transplantation; IED: immune effector dysfunction; MDS: myelodysplastic syndrome; ORR: overall response rate; OS: overall survival; PB: peripheral blood; R/R AML: relapsed or refractory acute myeloid leukemia; TCR: T cell receptor; Tregs: regulatory T cells; VAF: variant allele frequency.

employed by AML cells and T cells may be interrelated, as suggested by recent evidence collected in AML cell lines that induced expression of PD-L1 on AML cells could result in the conversion and subsequent expansion of CD4+CD25+FoxP3+ Tregs from CD4+ T cells[66].

CONCLUSION

In summary, through antigen recognition and co-stimulation, T cells front the adaptive immune response, causing AML cells to employ both cell-autonomous and cell non-autonomous mechanisms to create an immunosuppressive microenvironment and evade detection and killing by T cells. These mechanisms include (1) reduced expression of antigens and MHC molecules on the cell surface of AML cells; (2)

immune checkpoint activation to suppress T cell responses, both on T cells and on AML cells themselves; (3) induction of T cell exhaustion; and (4) the induction of an immunosuppressive environment by increasing the numbers of regulatory T cells and other immunosuppressive populations in the bone marrow to inhibit effector and cytotoxic T cell activity. All of these mechanisms ultimately promote AML cell survival. This review complements several other recent review articles in this field, which illustrate the importance of understanding the mechanisms of immune evasion in AML to overcome immunotherapy resistance and improve AML outcomes[13,53,67-69].

In our review of the current ICI landscape for hematologic malignancies, evident frustrations arise when comparing the success of checkpoint inhibitors in solid tumors to the more limited progress made with these agents in AML. The mechanisms highlighted above undoubtedly contribute to the slow adoption of ICIs in AML. With many clinical trials underway in this space, continued research efforts identifying ways to overcome immunotherapy resistance, such as combining ICIs with targeted therapies against components of signaling pathways notoriously activated in AML, as seen in solid tumors[70], are warranted. Furthermore, while not a major focus of this review, it remains a challenge to identify tumor-specific targets for personalized immunotherapies for AML, such as CAR T cells and bispecific antibodies[71-73].

While this review provides some insights into the roles of immune evasion mechanisms in relapse following stem cell transplantation, as well as the clinical trials underway utilizing ICIs for this patient population, the poor prognosis rates for AML patients who relapse after transplantation highlight the need for a review focused on this specifically. Some groups have taken this initiative already, including a summary of the current understanding of the downregulation of HLA molecules and inhibitory checkpoints between T cells and AML cells[74]. Additionally, recent insights into novel mechanisms by which an altered immune landscape following transplantation – characterized by increased expression of TIGIT and CD161 within the CD4+ T cell population post-transplantation – has begun to identify predictors of relapse[75]. A more recent review focuses on epigenetic mechanisms that underlie T cell evasion in the relapse post-transplant setting, and is also a good source for this topic[76].

Lastly, this review does not cover the advances and limitations of emerging immunotherapy treatment modalities in AML- notably chimeric antigen receptor (CAR) T- and NK-cell therapies, bispecific antibodies, dual affinity re-targeting (DART) molecules, monoclonal antibodies, and antibody-drug conjugates. While these agents are approved in other cancers [e.g., acute lymphocytic leukemia (ALL), non-Hodgkin lymphoma (NHL) subtypes, and multiple myeloma (MM)], their adoption in AML has been slow, due to the difficulty of finding AML-specific antigens that are not also expressed on HSCs or myeloid progenitors. Furthermore, mechanisms of antigen escape, the AML immunosuppressive environment, and the impaired quality of autologous cells are also potential problems with these approaches, as reviewed elsewhere[72]. Nonetheless, current clinical trials underway in relapsed/refractory AML include CD33, CD38, CD123, and CD19 CAR-T cell therapies, allogenic CAR NK-cells, and CD33xCD3 and CD123xCD3 bispecific antibodies[11,41].

Overall, as we continue to uncover the mechanisms underlying immune evasion in AML, exploiting these mechanisms will be of high priority to unleash the potential of immunotherapy in this disease. This is exemplified by the pivotal work done already, identifying a niche for immune checkpoint inhibitors after observing increased checkpoint expression in AML cells following HMA treatment[58]. Additionally, it will be important to identify strategies to suppress regulatory T cell activity in AML to allow for the unleashing of effector and cytotoxic and T cell activity. Thinking ahead, continued efforts to identify patient populations at higher risk for immune evasion during available treatments or following stem cell

transplantation, such as characterizing TIL populations prior to and during treatment, or examining T cell and NK cell numbers and function in specific molecular or cytogenetic subgroups of AML will pave the way for more personalized AML treatment plans.

DECLARATIONS

Authors' contributions

Wrote, reviewed, and edited the manuscript: Gurska L, Gritsman K.

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Conflicts of interest

Gritsman K has received research funding from ADC Therapeutics and iOnctura. Gurska L declares that there are no conflicts of interest.

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Not applicable.

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Not applicable.

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Cancer Drug Resistance

Review

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Drug resistance in glioblastoma: from chemo- to immunotherapy

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Abstract

As the most common and aggressive type of primary brain tumor in adults, glioblastoma is estimated to end over 10,000 lives each year in the United States alone. Stand treatment for glioblastoma, including surgery followed by radiotherapy and chemotherapy (i.e., Temozolomide), has been largely unchanged since early 2000. Cancer immunotherapy has significantly shifted the paradigm of cancer management in the past decade with various degrees of success in treating many hematopoietic cancers and some solid tumors, such as melanoma and non-small cell lung cancer (NSCLC). However, little progress has been made in the field of neuro-oncology, especially in the application of immunotherapy to glioblastoma treatment. In this review, we attempted to summarize the common drug resistance mechanisms in glioblastoma from Temozolomide to immunotherapy. Our intent is not to repeat the well-known difficulty in the area of neuro-oncology, such as the blood-brain barrier, but to provide some fresh insights into the molecular mechanisms responsible for resistance by summarizing some of the most recent literature. Through this review, we also hope to share some new ideas for improving the immunotherapy outcome of glioblastoma treatment.

Keywords: Glioblastoma, immunotherapy, drug resistance, tumor microenvironment, immunosuppression

INTRODUCTION

Brain tumors affect more than ~17,000 people in the United States each year, where gliomas are considered

the most common type of primary brain tumor[1]. Glioblastoma is a grade IV astrocytoma that was initially categorized into four molecular subtypes, termed neural, proneural, classical, and mesenchymal subtype[2]. Transcriptional profiling and genetic modeling in mice showed that glioblastoma originated from neural stem cells (NSC), NSC-derived astrocytes, and oligodendrocyte precursor cells (OPCs)[3-5]. Besides the four molecular subtypes based on their transcription profiling, glioblastoma tumors can also be classified by the status of the isocitrate dehydrogenase gene (IDH) as IDH wild-type and IDH-mutant tumors. Similarly, epigenetics factors, such as CpG island methylation phenotype of O6-methylguanine-DNA methyltransferase (MGMT) promoter, are also commonly used for glioblastoma tumor stratification[6,7].

Since the approval of Temozolomide (TMZ) for newly diagnosed glioblastoma treatment by the FDA in early 2000, surgery followed by radiotherapy and TMZ treatment has remained the first-line glioblastoma treatment[8]. However, none of these therapies eliminate cancer cells entirely because of challenges marred by high infiltration rate, tumor heterogeneity, blood-brain barrier (BBB), and immunosuppressive environment factors[9,10]. The highly infiltrative nature of glioblastoma does not allow the removal of cancerous cells using resection; self-renewing cells followed by resection become more prone to radioresistance and chemoresistance. Similarly, cellular heterogeneity and BBB prevent targeted drug delivery in glioblastoma[11,12].

COMMON DRUG RESISTANCE MECHANISMS IN BRAIN CANCERS

Blood-brain barrier

Although the BBB in glioblastoma is compromised to some extent, tumor BBB still presents a great challenge for therapeutics to reach glioblastoma cells. As the intrinsic barrier for brain cancer, BBB is a microvasculature structure surrounding the central nervous system (CNS), tightly regulating the movement of molecules and cells between the CNS and blood. Normally, BBB maintains the homeostasis of CNS and prevents infiltration of toxins, pathogens, inflammation, and harmful metabolites[13-15]. Disruption of the neurovascular unit (NVU) is associated with blood-brain dysfunction in neurodegenerative disease and brain tumors[16]. The NVU consists of vascular cells (endothelial, pericytes, and vascular smooth muscle cells), glia (astrocytes, microglia, and oligodendroglia), and neurons, and it plays an important role in maintaining BBB functional integrity and regulating the volume of cerebral blood flow[17,18]. The endothelial cells in neurovascular parenchyma form capillary beds connected through tight junctions (TJs), surrounded by a specialized basal lamina shared with pericytes and astrocytic end feet. They are sparsely interconnected by neuronal endings and microglia[19,20]. Astrocytes and pericytes, an essential constituent of NVU, release Sonic Hedgehog and vitronectin and angiopoietin I, respectively, acting on endothelial cells for their survival and maintaining BBB.

Overexpression of efflux pumps

Efflux transporters on the BBB membrane also contribute to cerebrospinal fluid homeostasis by protecting it from potentially harmful endogenous and exogenous substances[21,22]. These transporters also pose challenges by blocking therapeutic compounds from entering the brain parenchyma. Efflux transporters on compartments of the BBB belong to either ATP-binding cassette (ABC) or the solute carrier (SLC) superfamilies[23,24]. Organic anion-transporting polypeptides (OATP) are a superfamily of solute carrier organic anionic (SLCO) transmembrane transporters that are known for cancer drug resistance[25,26]. These peptide transporters regulate a variety of xenobiotic and endogenous substrates, including endogenous hormones, their conjugates, and anticancer drugs[27]. OATP1A2 is a sodium-independent uptake transporter family member and is highly expressed on the luminal membrane of BBB in tumors and adjacent healthy tissues[28]. A study by Cooper *et al.* in glioblastoma patients showed significant over-expression of all the OATP isoforms (OATP1A2, 2B1, 1C1, and 4A1) in tumor tissues compared to non-neoplastic brain[29].

Enhanced DNA damage repair pathways (MGMT) and abnormal activation of survival signaling pathways

As part of the glioblastoma standard treatment regimen, TMZ is a potent DNA alkylating agent that leads to DNA damage in cancer cells and cell death[30]. However, TMZ treatment often results in drug resistance in ~50% of glioblastoma patients due to overexpression of MGMT, which reverses the methylation of the O6 position of guanine. In addition to upregulated MGMT expression, glioblastoma often exhibits enhanced DNA damage repair capacity through several related mechanisms. For instance, poly(ADP-ribose) polymerase (PARP) was shown to interact with MGMT and enhance MGMT function in the removal of O6-methylation of DNA[31]. Interestingly, even in MGMT-deficient glioblastoma, TMZ resistance may still arise due to the loss of mismatch repair (MMR) pathway in tumor cells. Recent work by Lin *et al.* developed a new class of compound (KL-50) to achieve MMR-independent glioblastoma cell killing. It demonstrated a promising strategy to exploit cancer-specific deficiencies in DNA repair pathways[32]. Glioblastoma tumors also have elevated levels of receptor tyrosine kinases, such as EGFR gene amplification or mutation (EGFRvIII), PDGFR and FGFR, and aberrant activation of PI3K/ATK signaling and other growth factors (e.g., IGF-1, CTGF, and TGFβ)[33-39], with a potential contribution to the drug resistance phenotype.

Role of glioma stem cells

Glioma stem cells (GSCs) represent a subpopulation of relatively undifferentiated cells capable of self-renewal while also generating clonal populations of differentiated tumor cells in glioblastoma. These cells are increasingly recognized as a driving force supporting glioma genesis, therapy resistance, and recurrence[40]. GSCs have high regenerative capacity and can differentiate into cells expressing several lineage markers such as CD133, SOX2, CD15, CD44, integrin α6, and CD36[41]. Along with heterogeneity, various factors contribute to the chemoresistance of GSCs. Intrinsic factors include upregulated MGMT, higher anabolic capacity, and autophagy-mediated clearance of ROS induced by chemotherapy. Extrinsic factor is mainly hypoxic tumor microenvironment (TME). Hypoxia promotes the expression of GSC markers and a cancer stem-like phenotype[42]. Hypoxia-response genes, such as hypoxia-inducible factor HIF-2α and VEGF, are highly expressed in GSCs. Intriguingly, two reports have demonstrated that hypoxia-associated transcriptional signatures can be used as prognostic markers for glioblastoma patients[43,44].

Epigenetic modulations

Epigenetic dysregulation has been increasingly recognized as one of the significant drivers of oncogenesis, and several subtypes of glioblastoma are associated with epigenetic alterations[45,46]. These epigenetic modifications may serve as valuable biomarkers for tumor stratification and prognostic prediction. For instance, the glioblastoma resistance to receptor tyrosine kinase (RTK) inhibitors has been found to involve both genetic and epigenetic mechanisms[47], resulting in subclones with a gain of copy number in the insulin receptor substrate-1(IRS1) and substrate-2 (IRS2) loci. Another study identified a long non-coding RNA (LINC00021) that promotes TMZ resistance through Notch signaling and epigenetically silenced p21 expression via recruiting EZH2[48], one of the methyltransferases responsible for histone methylation. Epigenetic modifications in glioblastoma are also exploited as drug targets. Among the promising epigenetic interventions for glioblastoma are the histone deacetylase (HDAC) inhibitors[49], which have been extensively tested in various cancers[50]. HDAC inhibitors can block cancer cell proliferation by inducing cell cycle arrest, cell differentiation, and/or apoptosis[51]. With a large amount of supportive preclinical data, various HDAC inhibitors in glioblastoma clinical trials are underway.

DRUG RESISTANCE TO IMMUNOTHERAPY IN GLIOBLASTOMA

Current status of immunotherapy trials in glioblastoma

Although immune checkpoint inhibitors have greatly improved cancer treatment today, the clinical trials in glioblastoma treatment have been largely unsuccessful.

We summarized the most common immunotherapies that have been evaluated in glioblastoma in either preclinical or clinical trials [Figure 1]. The most widely tested immunotherapies in glioblastoma (like in all other cancers) are immune checkpoint inhibitors (ICIs). Immune checkpoint molecules are typically expressed on the surface of immune cells, and they play a crucial role in maintaining immune balance, preventing excessive immune activation, and avoiding auto-immune response. This function of immune regulation is achieved through the interaction of immune checkpoints with their corresponding ligands on other cells, and cancer cells often hijack this communication mechanism to suppress the anti-tumor immunity and evade immune surveillance[53-55]. A common working mechanism of ICIs is to block the inhibitory signal to the immune cells (usually from cancer cells) through an antibody binding to the checkpoint or its ligand to disengage their interaction. Since the discovery of the first immune checkpoint, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), more than a dozen of these checkpoint molecules have been identified to date, such as PD1 and its ligand PD-L1/L2, TIM3, lymphocyte activation gene-3 (LAG3), and TIGIT[54]. Among various ICIs, α-PD-1 has been widely studied as a monotherapy[56] or in a combination of either radiation or radiation plus TMZ in multiple trials (CheckMate 143, 498 and 548)[57-59]. Overall, the clinical outcome has been rather murky in both primary and recurrent glioblastoma due to multiple resistance mechanisms, including high tumor heterogeneity, low mutational burden, systemic immunosuppression, and local immune dysfunction[60].

CAR-T therapy has been studied in glioblastoma[61]. The targets of these CARs in clinical trials span from growth signaling receptors (EGFR/EGFRvIII, Her2), cytokine receptors (IL13Rα2), immune checkpoint (B7-H3) to even matrix metalloproteinase (MMP2), and disialoganglioside (GD2)[62]. Besides very limited responders, including pediatric patients with diffuse intrinsic pontine glioma (DIPG)[63,64], most trials failed to demonstrate a sustained clinical benefit, mainly due to CAR-T-associated severe side effects, including cytokine release syndrome and high grade of neurotoxicity[65,66].

Cancer vaccines have also been explored in glioblastoma trials with minimal success. A peptide vaccine targeting EGFRvIII called rindopepimut has been tested in various trials, with only one trial (phase II) reporting a marginal increase in median overall survival of 12.0 months with rindopepimut plus bevacizumab compared to 8.8 months with bevacizumab plus vaccine placebo[67]. The main limitation of EGFRvIII vaccine is that the expression of EGFRvIII is only limited in some glioblastoma patients, and there is also an intra-tumoral heterogeneous pattern of EGFRvIII expression, which further hinders the overall immune response to the tumor. Another cancer vaccine strategy is to use patient-derived dendritic cells with ex vivo exposure to glioblastoma neoantigens. For instance, ICT-107 and DCVax-L both used patient autologous dendritic cells with pulse to either peptides designed based on patient tumors (ICT-107) or autologous tumor lysates (DCVax-L). Both trials have reached phase 3 and had an acceptable safety profile, though the efficacy was minimal[68,69].

Oncolytic virus (OV) can be viewed as a gene & immuno-hybrid therapy. Typically, an OV exerts its anti-tumor function through a dual mode of action - tumor cell killing (lysis) and induction of systemic anti-tumor immunity[70]. An OV can selectively infect and lyse cancer cells, and various viruses have been employed to develop oncolytic viruses[71]. Upon lysis of tumor cells due to OV replication, many tumor antigens will be released, leading to a local and systemic anti-tumor reaction[72]. One of the main issues associated with OV therapy is the host's anti-viral immune response to the OV[73]. Currently, a modified herpes simplex virus type 1, named teserpaturev or G47Δ, is the only OV that received conditional approval (in Japan) for glioblastoma treatment[74], and many more oncolytic viruses are currently in clinical trials for glioblastoma treatment (reviewed by Suryawanshi & Schulze[75]). Among them, a retroviral OV called Toca511 reached phase III clinical trial, but was terminated due to its failure to improve survival and meet

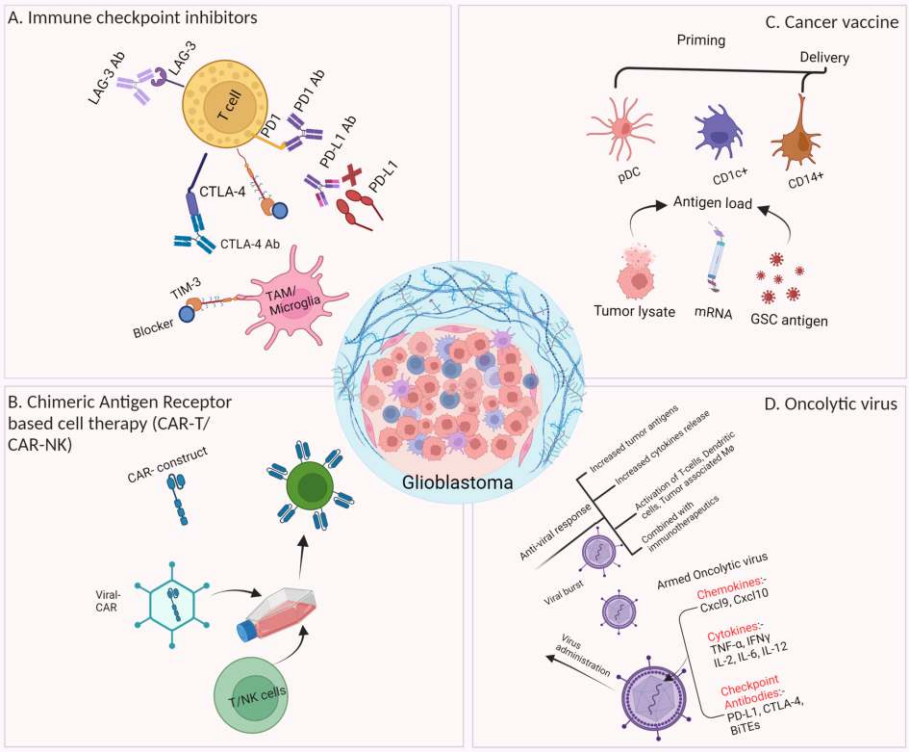

Figure 1. Various forms of immunotherapy in preclinical and clinical trials for glioblastoma treatment. (A) Various checkpoint inhibitors, including α-PD1, α-PD-L1, α-CTLA-4, and α-TIM-3, have been studied in glioblastoma treatment; (B) CAR-based adoptive cell therapies have attained immense success against hematopoietic cancer, but have shown limited effects on glioblastoma; (C) Cancer vaccine has been tested in glioblastoma treatment by priming antigen-presenting cells (e.g., Dendritic Cells) with tumor antigens/lysate or synthetic antigen peptides, followed by infusion back to the patients; (D) An OV can lyse tumor cells through replication. OV can be armed with immunotherapy in which a virus is genetically modified to carry checkpoint inhibitors (e.g., α-PD-L1 and α-CTLA-4), therapeutic proteins, chemokine (Cxcl9, Cxcl10) or cytokines genes (IFNγ, IL-6, IL-12). Those armed OVs are more potent in killing cancer cells[52]. (Created with BioRender.com). CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; GSC: glioma stem cell; LAG-3: lymphocyte activation gene-3; OV: oncolytic virus; TAM: tumor-associated macrophage.

other endpoints[75].

Immunosuppressive TME

Glioblastoma tumors generally have a low to moderate mutation rate, especially compared to other solid tumors such as melanoma, non-small cell lung cancer, GI cancer, and head and neck cancer[76]. The tumor mutation burden was found to be correlated with immunotherapy treatment response[77]. In addition, glioblastoma also has a highly immune-suppressive microenvironment with a large amount of infiltrating myeloid cells, including bone marrow-derived macrophages (MΦ), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and neutrophils[78]. T lymphocyte dysfunction in the glioblastoma is very severe and was found to be mediated partially by IL-10 produced by the myeloid cells[79]. Additionally, within the TME, prolonged antigen exposure to T cells leads to the expression of LAG3, which in turn causes T cell exhaustion[80]. More strikingly, patients with glioblastoma also have systemic immune suppression. For instance, glioblastoma patients have lower numbers of circulating T cells due to the sequestration of T cells in the bone marrow, possibly due to loss of sphingosine-1-phosphate receptor 1 (S1P1) expression[81]. S1P1 is a GPCR that binds the lipid second messenger, sphingosine-1-phosphate (S1P), and the S1P-S1P1 axis plays a pivotal role in lymphocyte trafficking[82]. Typically, surface S1P1 affords T cell egress from the spleen, lymph node, and thymus. In a mouse glioblastoma model, the T cells from tumor-bearing mice were found to have lost surface expression of S1P1, leading to T cells sequestered

mainly in bone marrow[81]. This may partially explain the T cell lymphopenia in glioblastoma patients. However, treatment (radiation and TMZ) associated T cell lymphopenia was also very common[83,84].

Glioblastoma tumors can produce IL-6 and drive myeloid immunosuppression by inducing PD-L1 expression on MDSCs[85]. Glioblastoma can also utilize the natural immune tolerance mechanisms to recruit regulatory T cells (Tregs) through the expression of indoleamine 2,3-dioxygenase (IDO)[86], as well as the tumor-associated macrophages (TAMs) expression of TIM4[87]. Besides soluble factors, extracellular vesicles containing various signaling molecules, including growth factors, non-coding RNAs, cytokines, and other functional proteins, have been found to play an important role in the regulation of glioblastoma TME[88]. Those mechanisms involve an extensive network of DCs, TAMs, MDSCs, and T lymphocytes with complex and dynamic crosstalk [Figure 2].

Heterogeneity in tumor microenvironment

Tumor heterogeneity has been well-known in glioblastoma biology at multiple levels[89], including genetics/epigenetics (molecular subtypes), molecular signaling (tumor driver mutations), cellular components (clonal and subclonal tumor cells *vs.* tumor microenvironment), and temporal (primary *vs.* secondary). scRNAseq analysis of infiltrating neoplastic cells in human glioblastoma revealed vast genomic and transcriptomic heterogeneity[90]. Another work in brain endothelial cells derived from human glioblastoma using a similar approach (scRNAseq) showed five distinct endothelial cell phenotypes representing different states of EC activation and BBB impairment and association with different anatomical locations within and around the tumor[91].

With the advancement of multi-omics platforms, tumor heterogeneity at both inter- and intra-tumoral levels has been much better depicted in glioblastoma[92-94]. The inter-tumoral heterogeneity can be readily appreciated by the molecular subtyping of human glioblastoma tumors by their transcriptional profile and phenotypical response to therapy[2,95,96]. Consistent with the four molecular subtypes of glioblastoma, a more recent scRNAseq analysis showed that glioblastoma cells can differentiate into four principal states, including astrocyte-like, oligodendrocyte progenitor-like, neural progenitor cell-like, and mesenchymal-like state[97]. These four cellular states are influenced by the tumor microenvironment and oncogenic drivers with certain plasticity[97].

The intra-tumoral heterogeneity in glioblastoma is characterized by the presence of clonal and subclonal differentiated tumor cells, glioma stem cells (GSCs), and various components of the tumor microenvironment (stromal, endothelial, and infiltrating immune cells). A recent study by Schaettler *et al.* using scRNAseq revealed the differences between primary and secondary glioblastoma in their genomic abnormality and neoantigen formation, as well as the spatially differential T cell clones within the glioblastoma[98]. The authors used TCR β-chain CDR3 sequences as unique barcodes of individual T cell clones, as TCR β-chain CDR3 is highly diverse with a significant role in antigen recognition[99]. Their results demonstrated a topological clonal diversity of T cells in glioblastoma[98]. Besides microglia, another representative cell population that further complicates glioblastoma heterogeneity is a large variety of myeloid cells in the TME[100]. They mainly comprise TAMs, MDSCs, DCs, neutrophils, and undifferentiated monocytes[78,101]. Another study using scRNAseq and multiplexing tissue-imaging techniques demonstrated a spatially differential tumor microenvironment characterized by inflammatory signaling and hypoxia in glioblastoma[102]. The authors revealed that CD73, a critical regulator of local purinergic signaling with an essential role in inflammatory response[103], was mainly expressed in glioblastoma cells with a positive correlation between levels of CD73 and HIF1α expression in the hypoxic tumor regions, where the CD73+ glioma cells co-localize with CD39+ microglia to form a spatially compartmentalized microenvironment to

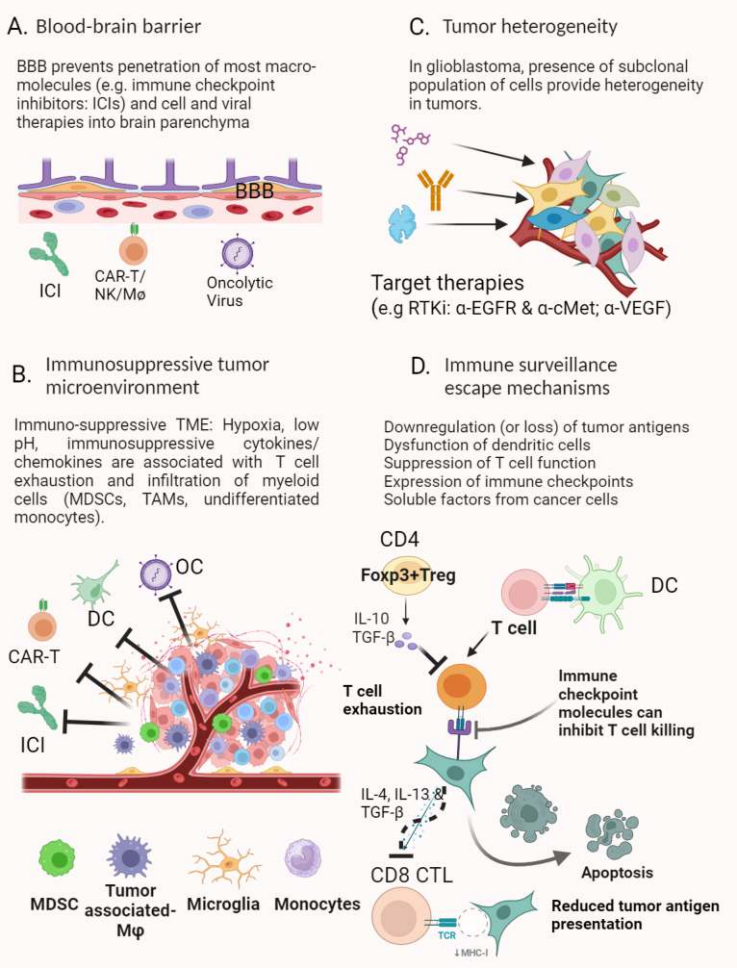

Figure 2. Main determinants of therapeutic failures in glioblastoma. (A) BBB can prevent the transport of most macromolecule therapeutics (e.g., immune checkpoint inhibitors), cell-based therapies, and most oncolytic viruses; (B) Within the glioblastoma, TME is a severely immunosuppressive local environment that can inhibit the function of most immunotherapies; (C) Clonal heterogeneity represents a complex problem for targeted therapeutics (e.g., receptor tyrosine kinase inhibitors and α-VEGF) to attack glioblastoma tumor cells effectively; (D) Various mechanisms for glioblastoma tumor cells to evade immune attack: tumor cells derived soluble factors (e.g., IL-4, IL-13, prostaglandin E2, and TGF-β) can suppress T cell proliferation; T cell exhaustion induced by prolonged antigen exposure can severely diminish CD8 CTL mediated cancer killing; FOXP3+ CD4 Tregs also block T cell activation. (Created with BioRender.com). BBB: Blood-brain barrier; CTL: cytotoxic T cell; DC: dendritic cell; ICIs: immune checkpoint inhibitors; MDSCs: myeloid-derived suppressor cells; TAMs: tumor-associated macrophages; TME: tumor microenvironment.

regulate the production of adenosine, a potent immunosuppressive metabolite[102].

Immune surveillance escape mechanisms

The crosstalk between glioblastoma and the TME through which glioblastoma tumors escape immune surveillance is very complex and highly dynamic, involving many signaling mechanisms, including both soluble factors and cell-cell interactions. Besides the BBB, which prevents drugs from reaching their target sites, these mechanisms include various immune-suppressive mechanisms, such as secretion of immunosuppressive cytokines (IL-10, TGF-β, and IL-6)[104,105], expression of immune checkpoints[106], and recruitment of regulatory T cells (Tregs)[107], induction of M2-like phenotype of tumor-associated MΦ and microglia[106], reduced tumor antigen presentation through downregulation of MHC expression, and the ability to evade immune through soluble ligands[108,109] [Figure 2].

T cell exhaustion

T cell exhaustion is exceptionally severe in glioblastoma[110], resulting in poor therapeutic efficacy of immunotherapy. Most immunotherapies focus on eliciting an anti-tumor T cell response that requires a collaboration of at least CD4 T Helper cells and CD8 cytotoxic T cells (CTLs). CD4 T Helpers can modulate antigen-specific immune response through their high plasticity and cytokine production, while CD8 CTLs exert cancer cell killing through direct cell-cell interaction and targeted release of effector molecules (perforin and granzymes)[111]. T cell exhaustion is mainly induced by persistent antigen exposure, and it is commonly seen in chronic infections and cancers. It is generally characterized by elevated expression of various immune checkpoints (PD-1, CTLA-4, LAG-3, and TIM-3). Remarkably, T cell exhaustion was also found to correlate with hypoxia in glioma, and both the number of exhausted T cells and the associated exhaustion markers (PD-L1, FOXO1, and PRDM1) correlated with HIF1α levels[112].

The presence of regulatory T cells (Tregs) is another contributing factor for the dysregulation of T cell function in glioma TME [Figure 2]. Tregs are a subset of CD4 T cells that usually prevent autoimmunity response via suppression of inflammation and maintenance of self-tolerance[113]. Tregs (CD4+ Foxp3+) naturally arise from thymic differentiation[114] or are induced in the already differentiated Foxp3- CD4+ T cells in the periphery[115]. A recent study showed that Tregs promote CD8 T cell exhaustion and restrict clonal diversity of tumor-infiltrating CD8 CTLs[116]. Therefore, strategies to eliminate Tregs have been developed to restore anti-tumor immunity in glioblastoma, including activation of glucocorticoid-induced tumor necrosis factor-related protein (GITR). GITR is an immune checkpoint constitutively expressed in Tregs, and its activation through ligand binding leads to the depletion of Tregs and reduced immunosuppression. A preclinical study by Amoozgar *et al.* demonstrated that targeting Tregs with anti-GITR antibodies can relieve resistance to immunotherapy (e.g., anti-PD1) in mouse glioblastoma models[117].

Immunosuppression by myeloid cells

A large number of myeloid cells, such as monocytes, macrophages and MDSCs, in the glioblastoma TME impose another great challenge for immunotherapy to function [Figure 2]. Among the tumor-infiltrating myeloid populations in glioblastoma, TAMs play a pivotal role in tumor progression, immunosuppression, and therapy resistance. TAMs are usually found to exhibit a tumor-promoting phenotype by producing immune suppressive cytokines such as IL-6, IL-10, and TGF-β[104,105], and they represent a large population of cells with immunosuppressive function in TME. Various approaches have been proposed to target TAMs for glioblastoma treatment. For instance, by dual targeting IL-6 and CD40, Yang *et al.* showed that they could reverse TAMs-mediated tumor immunosuppression and sensitize the glioblastoma tumor to immune checkpoint inhibitors (anti-PD1 and anti-CTLA-4) in mouse tumor models[118]. In addition, the relatively undifferentiated monocytic MDSCs have been found to play a significant role in glioblastoma-associated immunosuppression. Domenis *et al.* demonstrate that CD14+ monocytic MDSCs were the primary mediators of the T cell suppression induced by the GSC-derived exosomes containing various immune suppressive cytokines[119].

Glioblastoma can also evade immune attack by down-regulating tumor antigen expression. Tumor antigen loss during immunotherapy treatment, especially by CAR-T therapy, has been frequently reported[120]. Migrating or invading glioblastoma cells were found to have reduced expression of major histocompatibility complex (MHC) class I and II genes, resulting in significant down-regulation of tumor antigen presentation[121]. Additionally, glioblastoma TME is quite a hypoxic and acidic environment. Both hypoxia and acidosis are essential environmental cues for maintaining GSCs, especially in a HIF1α-dependent manner[122,123]. GSCs are believed to be primarily responsible for tumor resistance to chemotherapy and radiotherapy[124,125]. More importantly, GSCs have also been shown to have a significant role in the evasion of

immune function[126].

Resistance to ICIs

ICIs are currently the most prevalent immunotherapy for cancer treatment. Since the approval of the first ICI (α-CTLA-4) by the FDA in 2011, these antibodies have been studied in an increasingly growing number of clinical trials, including those cancers with low response rates, such as breast cancer, cervical cancer, and brain cancer[60,127,128]. Despite the success of ICIs in treating hematopoietic cancers, the clinical trials in glioblastoma have been underwhelming. Besides the BBB, several contributing factors that render ICIs ineffective in glioblastoma treatment have been identified.

Low tumor mutational burden in glioblastoma tumor

Glioblastoma is generally considered an immunologically "cold" tumor type with a relatively lower tumor mutational burden (TMB). Thus, the neoantigen levels are also lower[129,130]. Higher TMB often leads to the formation of a greater number of neoantigens and a greater potential for T-cell repertoire against tumor-specific antigens[131]. TMB has been found to be correlated with the clinical outcome of cancer immunotherapy[76]. Compared with the immunologically "hot" tumor types such as melanoma and NSCLC, glioblastoma shows a much lower neoantigen burden[132].

T cell dysfunction

Glioblastoma patients are often found to have T cell dysfunction in both CNS and peripheral blood, and T cell exhaustion is pervasive and severe in glioblastoma TME. CD8 T cell exhaustion usually starts with the loss of IL-2 production, a cytokine crucial for T cell proliferation, followed by loss or decreased production of TNF-α, IFN-γ, and granzyme B[133]. Tregs also make a significant contribution to the T cell dysfunction in glioma. Both natural and induced Tregs can suppress the cytotoxicity of CD8 CTLs. Tregs were found to be associated with worse prognosis in glioblastoma patients[134], and it seems that the natural Tregs are the dominant subpopulation of Tregs in glioblastoma. Besides dysregulated T cell function, surprisingly, neurons have been shown to play a role in the ICI therapy resistance in glioblastoma. A recent study reported neuronal calmodulin-dependent kinase kinase-2 (CaMKK2) as a driver for the resistance to ICIs in glioblastoma[56], in which CaMKK2 increased CD8 T cell exhaustion, reduced CD4 effector cell expansion, and played a role in the maintenance of immunosuppressive phenotype of tumor-associated microglia[135].

Deficits in antigen presentation by microglia

In glioblastoma TME, antigen presentation machinery is dysregulated in almost all types of antigen-presenting cells. The immunosuppressive microenvironment in glioblastoma leads to the downregulation of MHC expression in microglia[136,137]. The decreased MHC expression significantly impairs the ability of microglia to effectively present antigens, limiting the activation of other immune cells and undermining the immune response against the tumor. Similarly, TAMs were found to be deficient in antigen presentation, lacking costimulatory molecules CD86, CD80, and CD40 critical for T-cell activation[138]. In fact, although glioblastoma tumor-infiltrating dendritic cells seemed more efficient than both MΦ and microglia in priming T-cells with exogenous antigens[139], data from a preclinical study demonstrated that a better anti-tumor immunity is associated with both tumor-infiltrating dendritic cells and microglia[140].

TAMs

A new study using patient-derived recurrent glioblastoma tumors with neoadjuvant PD-1 antibody treatment showed that α-PD-1 activated T cells and dendritic cells, but was unable to reverse the immunosuppressive phenotype in TAMs[141]. Work by Chen *et al.* analyzed scRNAseq data from a combined of >19,000 individual macrophages from 66 human glioma cases (50 glioblastomas and 16 low-

grade gliomas) and discovered a pro-tumor subset of bone marrow-derived macrophages with the expression of a scavenger receptor MARCO[142]. More interestingly, this subpopulation of MARCO+ TAMs was found almost exclusively in the IDH-WT glioblastoma, and they exhibited a completely oppositive dynamic in α-PD-1 responders *vs.* non- responders[142]. Park *et al.* studied the immune landscape of mouse glioblastoma with α-PD-1 treatment, and found that chemokine CCL5 induced by α-PD-1 treatment seemed to recruit the anti-inflammatory TAMs into the glioblastoma TME[143]. A CyToF-based high-plexing immune profiling approach revealed that ICI-sensitivity in both human and mouse tumors was associated with a higher number of T cells and dendritic cells (DCs) and a lower number of PD-L1 positive TAMs[144].

Anti-inflammatory glucocorticoids

Glucocorticoids have been used to control certain adverse effects associated with cancer immunotherapy. Interestingly, concurrent administration of dexamethasone, a potent corticosteroid frequently used in glioblastoma patients to decrease tumor-associated edema, has been shown to be detrimental to immunotherapy for patients with glioblastoma[145]. Though the clinical data in this study was limited to a subset of patients with wild-type IDH-1 glioblastoma under α-PD-L1 treatment, the concurrent dexamethasone diminished the response to α-PD-1 therapy in two different mouse glioma models[145]. It is worth mentioning that glioblastoma patients under standard (radiation plus TMZ) treatment who received dexamethasone treatment also showed a worse outcome[146]. However, this is likely because MGMT promoter contains two nonconsensus glucocorticoid-responsive elements and glucocorticoids can upregulate MGMT expression[147]. A comprehensive study of MGMT promoter activity in glioblastoma cell lines further clarified that dexamethasone, but not TMZ or irradiation, can induce the upregulation of MGMT expression via a SP-1 dependent fashion[148], while not through altering the epigenetic status (i.e., methylation) of the MGMT promoter.

Role of non-coding RNAs

Long non-coding RNAs (LncRNAs) have been increasingly recognized for their essential role in cell growth, survival, proliferation, pluripotency, and immune functions correlating to the malignant transformation of normal cells into cancerous cells[149-151]. MALAT1, NEAT1, and H19 are among the common LncRNAs that influence the response of glioblastoma/glioma to chemotherapeutics[152]. Another lncRNA, LINC00021, was significantly upregulated in glioblastoma, especially in the TMZ resistance cells or tissues, enhancing resistance to TMZ through Notch pathway and epigenetically silencing p21 expression[48]. A study also showed that LncRNA SNHG15 promotes pro-glioblastoma cytokines TGF-β and lL-6 in TMZ-resistance cells via M2-polarization of microglial cells[153].

Micro RNA (miRNA) also plays a role in the regulation of glioblastoma TME. One example is the miR-15/16 cluster, which was found to be differentially expressed in various human cancers such as glioma and prostate cancer[154,155]. In a mouse glioblastoma model, Yang *et al.* demonstrated that loss of miR-15/16 in mice carrying GL261 tumors resulted in improved survival, enhanced CD8 T cell infiltration, and reduced expression of T cell exhaustion markers (PD1, TIM-3, and LAG-3)[156]. An *in vitro* study by Hubner *et al.* identified miR-93 as an anti-inflammatory tumor suppressor in glioblastoma[157]. Their data showed that miR-93 was downregulated in human glioblastoma cell lines, and restoration of miR-93 levels in glioblastoma cells led to a decreased expression of an array of inflammatory genes (HIF-1α, MAP3K2, IL-6, G-CSF, IL-8, LIF, and IL-1β)[157]. More interestingly, TCGA data mining confirmed that high expression of miR-93 was associated with better survival in the MGMT-methylated cohort of glioblastoma patients.

OPPORTUNITIES

Approaches to alter immuno-suppression in glioblastoma TME

Many great efforts have been made to overcome the difficulty of immunotherapy applications in neuro-oncology. For example, a clinical trial found that neoadjuvant PD-1 blockade resulted in significantly improved overall survival and progression-free survival in patients with recurrent glioblastoma[158]. In this study, patients received anti-PD1 treatment ~2 weeks before surgery, and the PD1 antibody was able to elicit both systemic and local anti-tumor immunity. Other attempts are primarily focused on modulating the immune suppression in the glioblastoma tumor microenvironment by targeting various components of the TME, such as TAMs and MDSCs (summarized in a recent review by Wang *et al.*[159]). In the meantime, new targets have been identified for future immunotherapy development. For instance, TAMs associated CD73 was found to be a promising target with potentially synergistic effects along with dual inhibition of PD1 and CTLA-4[160]. CD47/SIRPα axis is another exciting target to consider. SIRPα governs the phagocytosis activity of MΦ. When CD47 on the cancer cell surface engages with SIRPα on MΦ, it sends a "Don't-eat-me" signal to prevent phagocytosis of cancer cells by MΦ. Treatment with anti-CD47 plus TMZ was shown to activate both innate and adaptive anti-tumor immunity in a preclinical study[161].

A single-cell RNA-seq study of patient glioma infiltrating T cells revealed CD161 (KLRB1) as a promising immunotherapy target. Depleting CD161 led to T cell activation and anti-tumor immunity both *in vitro* and *in vivo*[162]. An independent study using data from a large cohort of glioma patients confirmed that CD161 might play an important role in promoting glioma progression via inhibition of T cell function[163].

Besides checkpoint inhibition, a deeper understanding of the resistance mechanism to CAR-T therapy in solid tumors was achieved through a genome-wide CRISPR knockout screen in glioblastoma[164]. A recent study using a genome-wide CRISPR knockout screen in glioblastoma revealed a functional requirement of IFN-γ receptor in glioblastoma for sufficient adhesion of CAR-T cells to mediate productive cytotoxicity[164]. This study suggests that strategies to enhance the binding of CAR-T cells to the solid tumor will likely result in a better treatment response. Another strategy to enhance the infiltration of CAR-T cells into glioblastoma tumors by combining CAR-T with a CXCL11-armed oncolytic virus also demonstrated an improved anti-tumor immunity in a syngeneic mouse glioma model[165].

Combinatorial approaches and new forms of immunotherapies

Combination therapy has been extensively explored to improve glioblastoma treatment. For instance, resistance to α-VEGF monotherapy was common in glioblastoma. A new study reported that combined blockade of VEGF, Angiopoietin-2, and PD1 could reprogram glioblastoma endothelial cells into quasi-antigen-presenting cells and induced a durable anti-tumor T cell response[166]. A recent review has nicely summarized the current status of combinatorial approaches, including both chemo- and immunotherapies, for glioblastoma treatment[167]. Additionally, many new forms of immunotherapy are emerging with great hope to shift the paradigm of glioblastoma treatment. A recent study reported a nanoporter (NP)-hydrogel complex for local induction of CAR-macrophages (CAR-MΦ) targeting CD133+ glioblastoma stem cells in tumor resection cavity with promising results[168]. This nanomicelle complex consists of a self-assembled peptide-based hydrogel loaded with the CD133-targeting CAR construct and then was coated with a citraconic anhydride–modified dextran with the ability to bind to CD206, a typical surface marker of M2 macrophages. Different from the *ex vivo* engineering of CAR-MΦ developed by Klichinsky *et al.*[169], the nanoporter-hydrogel-based in situ induction of strategy CAR-MΦ largely simplified the process of CAR-MΦ preparation and minimized potential systemic toxicity from CAR-MΦ.

The CAR-NK cells have also been explored to treat glioblastoma either by Her2 targeting monotherapy[170] or in combination therapy. For instance, the Off-the-Shelf EGFR-targeting CAR-NK cells have been tested in combination with an oncolytic virus expressing the IL15/IL15Ralpha complex and the combinatorial therapy demonstrates a strong anti-tumor immunity[171]. A significant problem associated with CAR-NK cell therapy is the shedding or down-regulation of the ligands in cancer cells that bind natural killer group 2D (NKG2D) receptors on the natural killer (NK) cells. NKG2D is an activating receptor widely expressed in NK cells as well as in some subsets of T cells[172]. To overcome the limitation of NKG2DL heterogeneity in the tumor, a recent study using a bispecific antibody with two ScFv fragments (linked with a IgG4-Fc) that target Her2 (tumor) and NKG2D (NK cells), respectively, in combination with human NK-92 cells, showed synergistic tumor cell killing effects in both *in vitro* and *in vivo* conditions[173]. Although the syngeneic tumor model they used represents a situation of a heterogenous expression of NKG2DLs in tumor cells, the flank tumors they used did not address the difficulty in delivery of the combination therapy across the BBB[173].

Another interesting phenomenon is the sex difference in response to immunotherapy in glioma. The sex disparity in brain cancer has been reported by several groups[174-177]. A recent meta-analysis revealed that female patients with glioblastoma treated with immunotherapy had a statistically significant survival advantage in overall survival over their male counterparts[178]. They also found that female patients exhibited a more robust survival advantage with cancer vaccine treatment. Another study by Bayik *et al.* discovered that two subsets of myeloid-derived suppressor cells (MDSCs) have a sex-specific tumor-promoting phenotype in both mouse and human glioblastoma[179]. All these data suggest that a more personalized approach, which at least considers sex differences in glioblastoma treatment, will more accurately evaluate the efficacy of immunotherapy.

New drug delivery technologies to overcome BBB limitation and activate glioblastoma TME

Various new technologies have demonstrated promising progress in overcoming BBB, and we summarized a few new approaches with great potential to improve the glioblastoma treatment outcome [Figure 3]. Among those new approaches, the use of ultrasound to open BBB for glioblastoma treatment has been applied in several areas, including immunotherapy delivery. Using low-intensity pulsed ultrasound to temporarily disrupt BBB, Sabbagh *et al.* demonstrated a significantly improved BBB penetration of both anti-PD1 antibody and EGFRvIII targeting CAR-T cells, as well as significantly improved survival in mouse glioblastoma models[180]. Another study by Sheybani *et al.* applied MRI-guided focused ultrasound with systemic injection of microbubbles and studied the impact of this approach on temporary BBB disruption in a mouse glioma model[181]. This approach caused a transient local inflammatory phenotype in the mouse glioblastoma, with an increased number of dendritic cells and the upregulated maturation marker. However, they did not see a significant increase in CD8 T cells in the TME[181].

Another technology to modulate BBB function is photodynamic therapy (PDT). Conventionally, PDT relies on a photosensitizer, such as 5-aminolevulinic acid (5-ALA)[182], that can accumulate in tumor tissue, plus a laser that can stimulate the photosensitizer, followed by energy transfer to generate reactive oxygen species, leading to damages to the cancer cells[183]. It is noteworthy that PDT has shown promise in temporary opening of BBB, possibly through modulating certain components of TJs[184]. Interestingly, PDT can also induce an acute inflammatory response in which both innate and adaptive immune systems are activated[185]. Recently, BBB opening was shown to affect the meningeal lymphatic system characterized by an anti-tumor effect of talaporfin sodium (TS)-PDT as well as its synergy with the immune checkpoint inhibitor[186]. *In vitro* studies have demonstrated that targeted TS-PDT triggers various forms of cell death, including apoptosis, necrosis, and autophagy-associated cell death. Furthermore, TS-PDT induces the acute activation of lymphatic drainage in the brain and the clearance of unwanted molecules from the CNS[187,188].

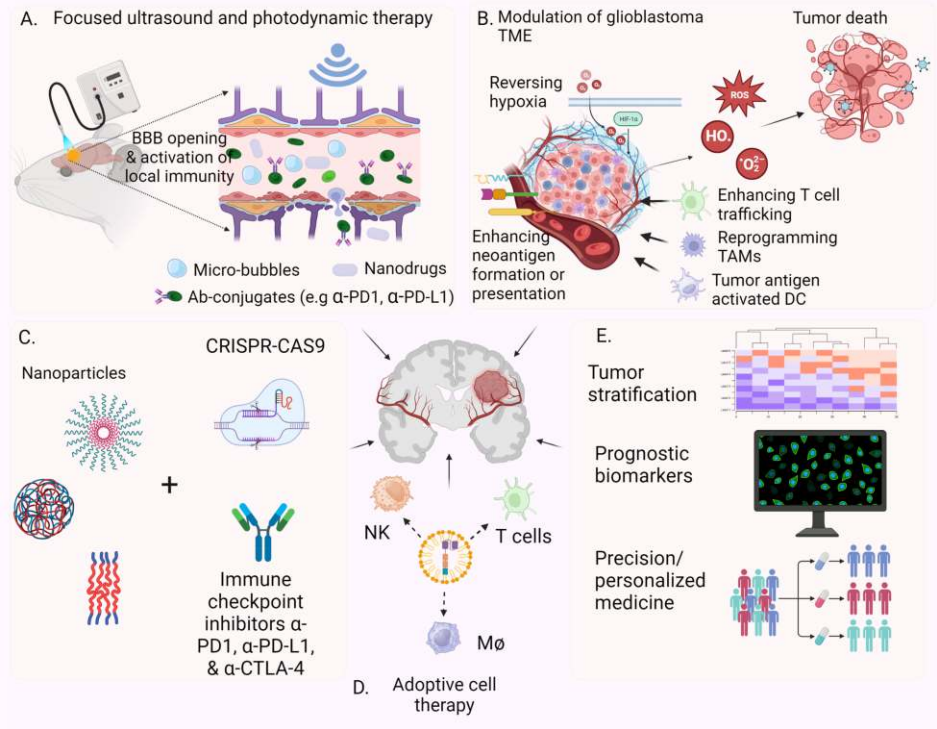

Figure 3. Potential new approaches to improve glioblastoma treatment. (A) The focused ultrasound in combination with micro-bubbles and photodynamic therapy (PDT) can temporarily open BBB to allow therapeutics crossing. PDT can also activate local immunity in TME; (B) New approaches to modulate glioblastoma TME by targeting hypoxia, activating suppressed local immunity, or enhancing cancer neoantigen formation in tumor cells; (C) Novel nanodrug delivery technologies in combination with CRISPR/Cas9-based gene editing and immune checkpoint inhibitors; (D) Various forms of adoptive cell therapies; (E) Better strategies for tumor stratification, prognostic prediction and personalized medicine would enhance the clinical outcome of glioblastoma treatment. (Created with BioRender.com). BBB: Blood-brain barrier; DC: dendritic cell; TAMs: tumor-associated macrophages; TME: tumor microenvironment.

The approval of 5-ALA by the FDA for fluorescence-guided glioblastoma resection has sparked a renewed interest in its potential application for PDT[182].

Nanotechnology has also made significant advancements in the field of glioblastoma treatment. Various forms of nanomedicines have exploited the features of the glioblastoma tumor microenvironment for efficient BBB crossing and release of payloads[189-191]. Fan *et al.* engineered an MMP-2-activated nanoparticle to carry anti-CD276 & CD3 bispecific antibodies and demonstrated that this strategy enhanced IFN-γ-induced tumor cell ferroptosis[192]. A polylactic-co-glycolic acid (PLGA) nanoparticle encapsulated disulfiram was used to block hypoxia-induced NF-κB signaling and glioma stem cells[193]. Zou *et al.* devised a polymer-based CRISPR-Cas9 nano-capsule for systemic gene therapy delivery to glioblastoma[194]. This nano-capsule has both the BBB crossing and tumor targeting functions mediated through an angiopep-2 peptide[195]. By targeting polo-like kinase (PLK-1) via a sgRNA, the strategy demonstrated a significant survival advantage over the control mice[194].

CONCLUSION

Despite advances in surgical technologies and therapeutics development, there has been limited improvement in the long-term survival rate of glioblastoma patients, with a 5-year survival still around 5%-10%. Many lessons have been learned in glioblastoma drug resistance mechanisms, especially with cutting-edge scRNAseq, spatial biology, and other-omics platforms. Efforts are needed to overcome BBB and tumor heterogeneity, targeting glioma stem cells and their niches, enhancing T cell trafficking and preventing their

exhaustion, and modulating the immunosuppressive TME in glioblastoma. A complex disease, such as glioblastoma, would require a complex solution. Multidisciplinary approaches involving nanodrug carriers, focused ultrasound, plus temporary BBB permeability enhancement technologies (micro-bubbles, phototherapy) in combination with gene and immuno-therapy will likely lead to an improved outcome [Figure 3]. In addition, a much less traveled path is to enhance glioblastoma neoantigen formation. Glioblastoma tumors have a relatively lower TMB, which was shown to correlate with immunotherapy outcomes in solid tumors[76,196]. Lower TMB results in lower neoantigen generation, which enables a stealth mode of glioblastoma cells. Therefore, increasing the formation of neoantigens may significantly promote tumor recognition and clearance by the immune system[197]. Besides T cells, strategies to activate other infiltrating immune cells (TAMs, microglia, and MDSCs) that reside in the glioma TME in large abundance may effectively reverse the local immunosuppression. Finally, a more precise tumor stratification approach and improved prognostic biomarkers will help determine the most effective combinatorial therapies for glioblastoma treatment.

DECLARATIONS

Authors' contributions

Conceptualization, investigation, writing: Sharma S, Chepurna O
Conceptualization, supervision, writing: Sun T

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Cancer Drug Resistance

Original Article

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Establishment of head and neck squamous cell carcinoma mouse models for cetuximab resistance and sensitivity

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Abstract

Aim: Acquired resistance to the targeted agent cetuximab poses a significant challenge in finding effective anti-cancer treatments for head and neck squamous cell carcinoma (HNSCC). To accurately study novel combination treatments, suitable preclinical mouse models for cetuximab resistance are key yet currently limited. This study aimed to optimize an acquired cetuximab-resistant mouse model, with preservation of the innate immunity, ensuring intact antibody-dependent cellular cytotoxicity (ADCC) functionality.

Methods: Cetuximab-sensitive and acquired-resistant HNSCC cell lines, generated *in vitro*, were subcutaneously engrafted in Rag2 knock-out (KO), BALB/c Nude and CB17 Scid mice with/without Matrigel or Geltrex. Once tumor growth was established, mice were intraperitoneally injected twice a week with cetuximab for a maximum of 3 weeks. In addition, immunohistochemistry was used to evaluate the tumor and its microenvironment.

Results: Despite several adjustments in cell number, cell lines and the addition of Matrigel, Rag2 KO and BALB/C Nude mice proved to be unsuitable for xenografting our HNSCC cell lines. Durable tumor growth of resistant SC263-R cells could be induced in CB17 Scid mice. However, these cells had lost their resistance phenotype *in vivo*. Immunohistochemistry revealed a high infiltration of macrophages in cetuximab-treated SC263-R tumors. FaDu-S and FaDu-R cells successfully engrafted into CB17 Scid mice and maintained their sensitivity/resistance to cetuximab.

Conclusion: We have established *in vivo* HNSCC mouse models with intact ADCC functionality for cetuximab resistance and sensitivity using the FaDu-R and FaDu-S cell lines, respectively. These models serve as valuable tools for investigating cetuximab resistance mechanisms and exploring novel drug combination strategies.

Keywords: HNSCC, cetuximab resistance, xenograft mouse model, immunodeficient mice

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is a type of cancer originating in the mucous membranes of the oral cavity, pharynx, and larynx. With over 800,000 patients being diagnosed every year[1], HNSCC remains a challenging disease to treat. Over the years, novel treatment options have emerged targeting the tumor in a much more specific way, thereby reducing unwanted side effects associated with more conventional therapies, such as chemo- and radiotherapy. These novel immuno- and targeted therapies include the anti-programmed cell death-1 (PD-1) monoclonal antibody (mAb) pembrolizumab and the anti-epidermal growth factor receptor (EGFR) mAb cetuximab, which are now, respectively, first- and second-line therapy for the treatment of recurrent/metastatic (R/M) HNSCC[2-4]. In addition, the PD-1 antibody nivolumab was already approved in 2016 for the treatment of patients with R/M HNSCC who have progressed on or after platinum-based chemotherapy[5]. Although these therapies have proven their worth in terms of prolonging survival and tolerability, the development of therapeutic resistance, leading to a lack of durable efficacy, is a major roadblock in the search for effective treatment options in HNSCC. Finding a way to overcome this resistance might contribute to the much-needed progress in the field. Based on our own previous, extensive research on cetuximab resistance[6-9] as well as preclinical and clinical studies reported in literature[10-12], inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway might be a promising therapeutic strategy to increase response to EGFR blockade with cetuximab. Recently, we demonstrated that the addition of the Akt inhibitor MK2206 to cetuximab treatment resulted in synergistic effects in both cetuximab-sensitive and acquired cetuximab-resistant HNSCC cell lines[8]. However, *in vivo* validation of this combination strategy to overcome cetuximab resistance is yet to be investigated. Although cell lines grown in simple two-dimensional (2D) culture systems are useful and informative for initial screening of anti-cancer drugs, *in vivo* evaluation is essential for several reasons. Firstly, *in vivo* studies can provide information on the pharmacokinetics and toxicity of a drug, which cannot be accurately predicted by *in vitro* tests. Secondly, *in vivo* studies can reveal the efficacy of a drug in reducing tumor growth and metastasis, which is ultimately the most important factor in determining its clinical utility. Lastly, and maybe the most important reason of all, 2D culture systems do not accurately replicate the complex microenvironment of a tumor, including interactions with stromal cells, immune cells, endothelial cells, and the extracellular matrix (ECM). In this context, it has already been shown that cell polarity, nuclear organization, and gene expression in tumor cells are affected by their interaction with the ECM[13]. In addition, tumor cells grown in three-dimensional (3D) culture systems exhibit clear differences in growth characteristics and response to chemotherapeutics compared to cells grown in conventional cell culture systems[14-16]. Over the past few years, significant progress has been made

in developing more advanced culture systems that are able to more closely resemble the patient's original tumor. For example, 3D tumor organoid cell culture models co-cultured with autologous immune cells or stromal cells have already been demonstrated to be a useful tool for studying the interaction of tumor cells with the tumor microenvironment (TME)[17,18]. Moreover, organ-on-chip technology has emerged as an innovative approach that integrates multiple cell types and can simulate the cellular and biochemical processes occurring in the TME[19]. Furthermore, 3D organotypic co-culture models have proven effective in maintaining the architecture and cell composition of the original tumor[20]. Interestingly, a 3D collagen-based scaffold model has been demonstrated to be a valuable tool for studying the TME and therapeutic resistance mechanisms in HNSCC[21]. Despite these advancements, it is important to acknowledge that current cell culture systems still have limitations in fully capturing the complexity of tumors and their interactions with the TME. As such, *in vivo* evaluation remains a crucial step in the drug development process and is necessary to ensure the safety and efficacy of novel treatment combination strategies, including cetuximab, before they can be approved for clinical use. However, to date, the availability of adequate *in vivo* mouse models specifically designed to study cetuximab resistance and sensitivity remains limited.

Historically, the working mechanism of cetuximab has largely been attributed to the direct effects of EGFR inhibition. However, cetuximab is also involved in processes that stimulate the immune system[22-24]. In this regard, cetuximab, being a chimeric human:mouse immunoglobulin G1 (IgG1), is able to mediate cellular immunity by inducing antibody-dependent cellular cytotoxicity (ADCC)[25,26]. This is a biological process where the fragment crystallizable (Fc) region of the antibody can bind to CD16 Fc receptors located on natural killer (NK) cells, macrophages and granulocytes, with NK cells being the most potent effectors[27]. This Fc-CD16 binding triggers the release of cytolytic proteins such as granzymes and perforin, leading to targeted destruction of tumor cells through apoptosis or lysis[23,28]. Moreover, studies have demonstrated that cetuximab has the ability to promote cross-priming of cytotoxic T cells via antigen-presenting cells such as dendritic cells[29]. This effect is primarily attributed to the induction of immunogenic cell death by cetuximab in tumor cells[30]. As such, the immune-mediated effects of cetuximab play a significant role in its antitumor activity. Therefore, in this study, we aimed to optimize two mouse models, with different cetuximab resistance status and intact ADCC functionality, that are able to subcutaneously grow tumors from human cell lines proven to be cetuximab-sensitive and -resistant *in vitro* and *in vivo*. Preservation of ADCC functionality in these mouse models is crucial to ensure that cetuximab can still execute not only EGFR inhibition but also mediate ADCC as part of its antitumor effects. Although partially immunodeficient, these mouse models are more representative of the human situation, since they are capable of executing ADCC, potentially improving the translatability of cetuximab responses from mice to humans. These two mouse models can be used in the future to test the potency of novel combination strategies containing cetuximab with the goal of overcoming resistance to cetuximab and exploring cetuximab resistance mechanisms in an *in vivo* setting.

METHODS

Cell lines and cell culture

We included three sets of isogenic cetuximab-sensitive versus acquired -resistant HNSCC cell lines. The SC263 cell line was kindly provided by Prof. Dr. Sandra Nuyts (University Hospital Leuven, Leuven, Belgium), the SCC22b cell line was kindly provided by Prof. Dr. Olivier De Wever (Laboratory of Experimental Cancer Research, Ghent University Hospital, Ghent, Belgium) and the FaDu-S and FaDu-R cell lines were kindly provided by Prof. Dr. Cyril Corbet (Pole of Pharmacology and Therapeutics, Institut de Recherche Expérimentale et Clinique, UCLouvain, Brussels, Belgium). Acquired-resistant variants (suffix R) of the initially cetuximab-sensitive SC263 and SCC22b cell lines were generated by chronic exposure to cetuximab as described previously by us[7]. In parallel, parental cell lines were exposed to the vehicle control,

i.e., phosphate-buffered saline (PBS), and used as a control for vehicle exposure and an increased culture period (suffix S). Before inoculation, acquired-resistant cell lines were exposed to a high dose of cetuximab for 7 days to ensure proper selection of resistant cells. All cell lines were human papilloma virus (HPV)-negative and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco™, 10938025), supplemented with 10% fetal bovine serum (FBS, Gibco™, 10270106), 1% penicillin/streptomycin (Gibco™, 15140122), and 2 mM L-glutamine (Gibco™, 25030024). Cells were grown as monolayers and maintained in exponential growth in 5% CO_2/95% air in a humidified incubator at 37 °C. Cell lines were confirmed free of mycoplasma infection through regular testing using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-118). The identity of each cell line was validated through short tandem repeat profiling.

Animal facilities and animals

All animal care and testing were approved by the Ethics Committee of the University of Antwerp (N° 2020-41 and 2021-39) and performed according to the European guidelines within the facilities of the University of Antwerp, Campus Drie Eiken.

Female C57BL/6NRj-Rag2tm1Ciphe/Rj [Rag2 knock-out (KO)] mice, aged 4-6 weeks, were obtained from Janvier Labs. Female BALB/cAnN.Cg-Foxn1nu/Crl (BALB/c nude) and CB17/lcr-PrkdcScid/lcrlcoCrl (CB17 Scid) mice, aged 4-6 weeks, were obtained from Charles River Laboratories (supplier of The Jackson Laboratory in Europe). After arrival, mice were allowed to acclimatize for at least 7 days before being used in experiments to reduce stress levels. All mice were housed in controlled, specific pathogen-free environments with 12-hour cycles of light and dark and provided with food and clean water ad libitum. Mice were monitored daily for humane endpoints (body weight, appearance, behavior, and comorbidities). The number of mice varied through the experiments, taking into account both feasibility and ethical considerations. Adjustments were made based on the outcome of previous experiments, ensuring meaningful conclusions while minimizing the use of animals. The principle of reduction, refinement, and replacement (3Rs) was followed, leading to the use of minimal numbers in all experiments.

Tumor kinetics and survival

Prior to injection, tumor cells were harvested using TrypLE (Gibco™, 12604021) and washed 3 times with sterile Dulbecco's phosphate-buffered saline (DPBS, Gibco™, 14190144). In all experiments, tumor cells were suspended in 100 µL sterile PBS and injected into the shaved hind flank of the mice. In each experiment, mice were injected subcutaneously with cetuximab-sensitive or -resistant HNSCC cells at different concentrations and with/without Matrigel or Geltrex according to the schematic overview in Figure 1. When tumors reached a size of approximately 30 or 70 mm^2, mice were randomized based on tumor size and divided into different treatment groups. Tumor growth was monitored over time and measured two times a week using a digital caliper. Tumor size was calculated using the formula "length × width". Mice were sacrificed when a tumor size of 150 mm^2 was reached or when a humane endpoint was reached [Figure 1].

In vivo administration of cetuximab

Mice were treated twice per week (with an interval of 3 to 4 days) with a low (2.5 mg/kg), medium (10 mg/kg), or high (50 mg/kg) dose of the anti-EGFR-targeted mAb cetuximab (Merck) or PBS control through intraperitoneal (i.p.) injection at the contralateral abdominal side of the tumor for a total treatment duration of 3 weeks. The doses (2.5, 10, and 50 mg/kg) were determined based on literature[31-34]. Iida *et al.* reported that no discernible toxicity was observed in mice treated with 50 mg/kg cetuximab twice a week for 10 consecutive weeks[33]. Calculations of the required cetuximab concentration in mg/kg were made for each individual mouse based on the individual body weight.

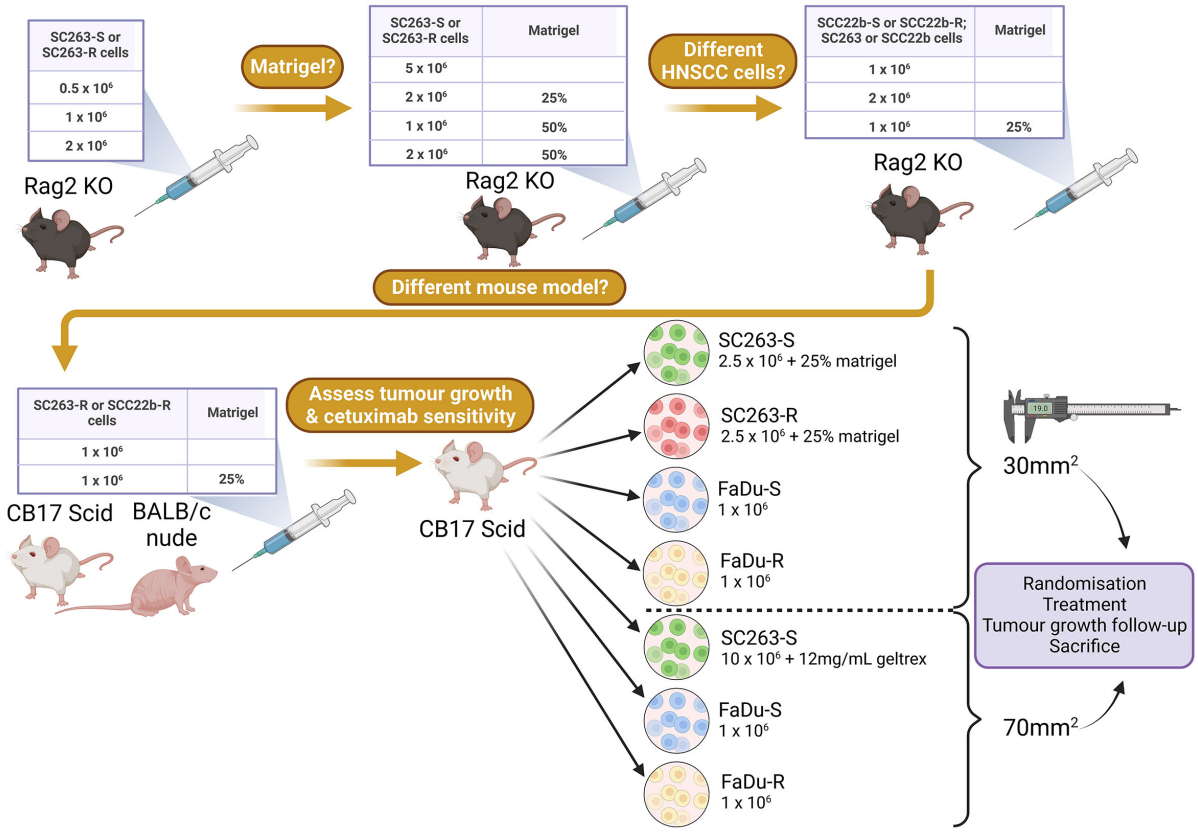

Figure 1. Schematic overview of performed experiments. This figure was created with Biorender.com. HNSCC: Head and neck squamous cell carcinoma; KO: knock-out; -R: cetuximab-resistant HNSCC cell line; -S: cetuximab-sensitive HNSCC cell line.

Immunohistochemical analysis

SC263-R, FaDu-S, and FaDu-R tumors were harvested directly after the last week of cetuximab treatment (or before tumors disappeared completely), whereas tumors originating from the SC263-S cell line were collected without prior treatment, since inducing a durable tumor growth was challenging with this cell line. Formalin-fixed paraffin-embedded (FFPE, 5 µm thick) sections were prepared from tumor tissue blocks. Sections were incubated in a low pH buffer (pH6) for 20 min at 97 °C (PT-Link, DAKO) for heat-induced antigen retrieval. Peroxidase blocking buffer (3.5%, Acros Organics, 202465000) was used for 10 min to quench the endogenous peroxidase activity of the sections, followed by blocking with normal goat serum (for anti-F4/80, anti-Ki67 and cleaved caspase-3) or normal horse serum (for anti-NKp46). Subsequently, sections were incubated with primary antibodies: anti-NKp46 [1:50 for 60 min, NK cell marker, Bio-Techne (R&D Systems), polyclonal, AF2225-SP], anti-F4/80 (1:500 for 40 min, macrophage marker, Thermo Fisher Scientific, clone BM8, 14-4801-81), anti-Ki67 (1:400 for 35 min, proliferation marker, Cell Signaling Technology, clone D3B5, 12202S), and anti-cleaved caspase-3 (1:250 for 35 min, apoptosis marker, Cell Signaling Technology, polyclonal, 9661S). The ImmPRESS™ goat anti-rabbit peroxidase kit (for anti-Ki67 and cleaved caspase-3, Vector, MP-7451), the ImmPRESS™ goat anti-rat peroxidase kit (for anti-F4/80, Vector, MP-7444), or the ImmPRESS™ horse anti-goat peroxidase kit (for anti-NKp46, Vector, MP-7405) in combination with the liquid DAB+ substrate chromogen system (DAKO, K3467) were used for signal detection according to the manufacturer's instructions. All sections were counterstained with hematoxylin (0.1%, Merck, C.I.75290), dehydrated in a series of isopropanol baths (distilled water, 70%, 95%, 100%, Acros Organics, P/7490/FP21), cleared with xylene (MLS, ZY10020) and mounted with ExPert mounting medium (MLS, QC50082). Positive controls were included for each marker and consisted of mouse tissue of

spleen (for anti-Ki67, anti-F4/80, and anti-NKp46) and lymph node (for anti-cleaved caspase-3). Pictures were taken with a Leica ICC50 E camera on a Leica DM500 microscope.

Statistical analysis

Possible significant differences in tumor kinetics between treatment groups ($P < 0.05$) were evaluated with a linear mixed model by each time point with the treatment group as a fixed effect and the subject as a random effect using JMP Pro v16.0.0 software.

RESULTS

Rag2 KO mice prove to be ineffective as xenograft models for HNSCC tumor growth

Rag2 KO mice were selected as a suitable model for our first experiment, since this mouse strain has no mature B and T cells and is considered an excellent xenograft host for cancer cell lines. Importantly, the innate immunity is still intact in these mice. Thus, this model is highly suitable for testing the efficacy of therapeutic antibodies, such as cetuximab, as ADCC is still intact.

Rag2 KO mice were injected subcutaneously with a low (0.5×10^6), medium (1×10^6), and high (2×10^6) number of the cetuximab-sensitive SC263-S and acquired cetuximab-resistant SC263-R cells and tumor growth was followed up over time. The SC263-S and SC263-R cells were selected, as these cell lines showed the most promising responses in *in vitro* combination experiments. Unfortunately, after 4 weeks of follow-up, no mouse had developed a measurable subcutaneous (s.c.) tumor. Therefore, we tried to achieve tumor growth by varying several conditions. Firstly, we co-injected the tumor cells with Matrigel (25% and 50%) since, according to literature, Matrigel co-injection can increase the initiation and growth of tumor cells *in vivo*[35]. In addition, a group of mice inoculated with 5×10^6 cells (without Matrigel) was included, as some HNSCC xenograft studies using such high cell numbers have been described in literature[36-38]. After 2 weeks of follow-up, only the Matrigel groups showed measurable s.c. tumors that persisted for multiple measurements (≥ 3, Figure 2). However, some tumors completely disappeared over time, while most tumors of both cell lines slowly regressed, reaching a plateau without further exponential growth, and tumor sizes never reached 30 mm^2 [Figure 2]. As a result, we decided to terminate this experiment, as it became evident that none of the groups would be appropriate for conducting further experiments.

Considering that the in-house developed SC263-S and SC263-R cell lines, as well as the parental SC263 cell line, have not been previously used in xenograft models to our knowledge, the results we obtained suggest that these cell lines may not be capable of initiating tumor growth *in vivo*. To further explore this hypothesis, we repeated the previous experiment with the SCC22b-S and SCC22b-R cell lines, with/without co-injection with Matrigel, as the parental SCC22b cell line has been employed in HNSCC xenograft studies before[39-42], yet in other mouse models than Rag2 KO. Unfortunately, neither experimental group exhibited any sustainable tumor growth, indicating that these cell lines were also unsuitable as progressive HNSCC models in the Rag2 KO mouse strain. Although tumor growth was initially observed in mice injected with 2×10^6 SCC22b-S cells with 25% Matrigel, this was very limited (tumor sizes < 17 mm^2) and tumor size decreased rapidly over time [Figure 3]. To eliminate the possibility that the increased passage numbers of these cell lines due to in-house development of acquired resistance were causing the issue, we tested the parental SC263 and SCC22b cell lines in the Rag2 KO mouse strain with/without Matrigel, but without any success. In conclusion, we attempted several methods, including co-injection with Matrigel, adjusting cell number, multiple cell lines and testing parental cell lines, but none of them proved effective. These results led to the conclusion that the Rag2 KO mouse strain is unsuitable for xenografting HNSCC cell lines, at least for our sets of isogenic cetuximab-sensitive versus acquired -resistant cell lines.

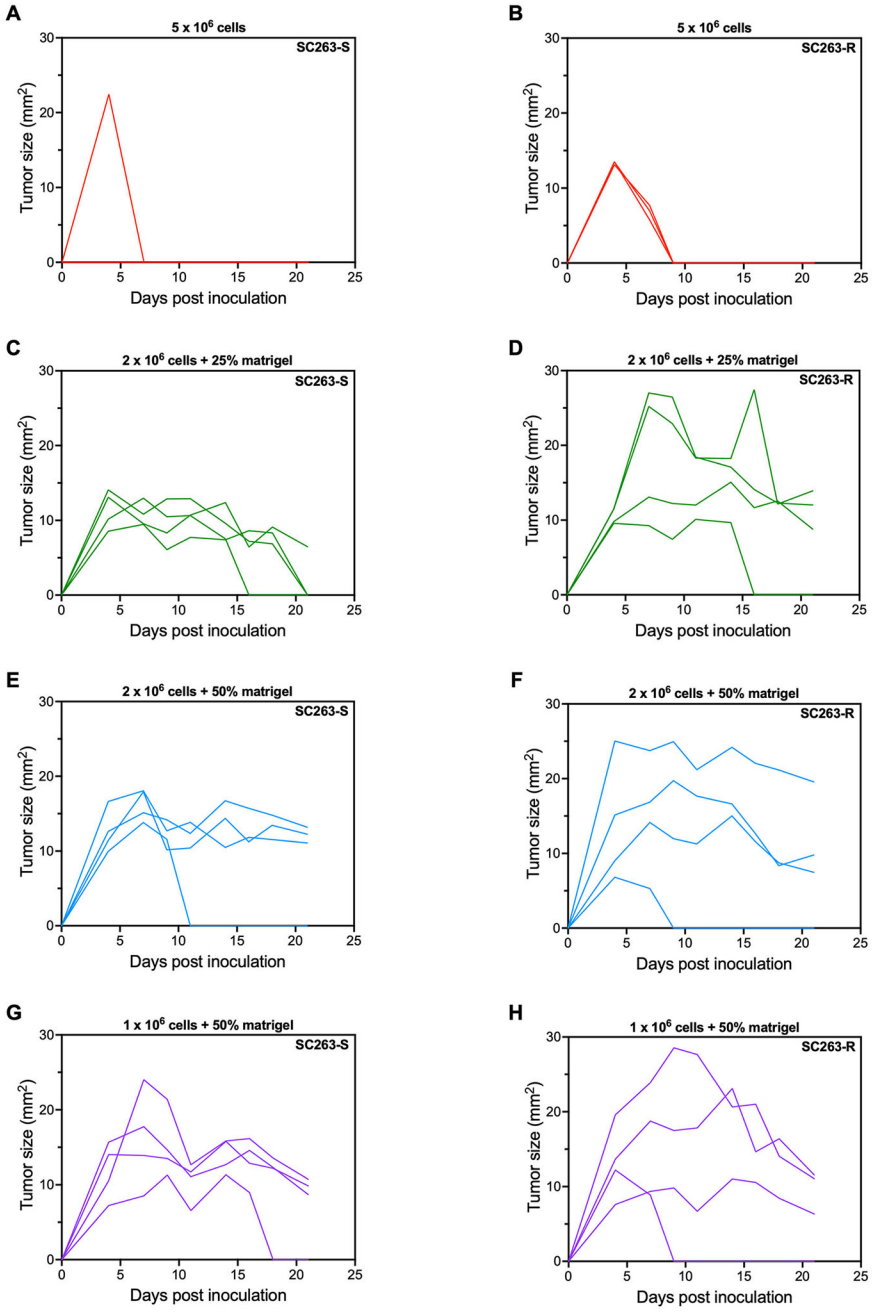

Figure 2. Co-injection of SC263 tumor cells with Matrigel in Rag2 KO mice did not result in sustained tumor growth. (A and B) Tumor kinetics after s.c. injection with 5 × 10^6 SC263-S: (A) and SC263-R cells; (B) (n = 3); (C and D) Tumor kinetics after s.c. injection with 2 × 10^6 SC263-S: (C) and SC263-R cells; (D) mixed with 25% Matrigel (n = 4); (E and F) Tumor kinetics after s.c. injection with 2 × 10^6 SC263-S: (E) and SC263-R cells; (F) mixed with 50% Matrigel (n = 4); (G and H) Tumor kinetics after s.c. injection with 1 × 10^6 SC263-S: (G) and SC263-R cells; (H) mixed with 50% Matrigel (n = 4). Each line represents the data of one individual mouse. KO: Knock-out; -R: cetuximab-resistant HNSCC cell line; -S: cetuximab-sensitive HNSCC cell line; s.c.: subcutaneous.

CB17 Scid mice prove to be a suitable host for resistant SC263-R HNSCC cells

In a subsequent pilot experiment guided by literature and advice from Janvier Labs, we evaluated the suitability of two different immunodeficient mouse models for xenografting human HNSCC cancer cell lines. More specifically, we injected BALB/c nude and CB17 Scid mice with SC263-R cells, both with and

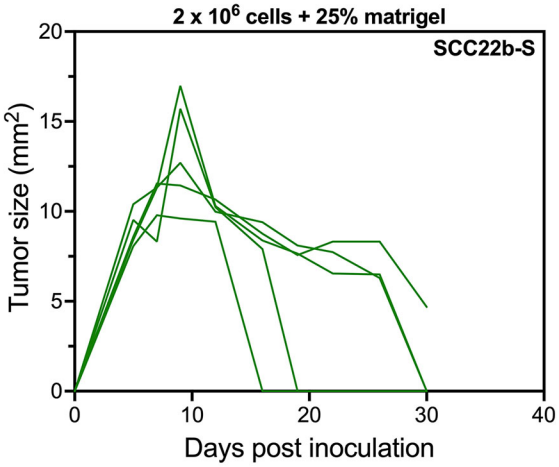

Figure 3. Tumor kinetics of SCC22b-S HNSCC cells with 25% Matrigel in a Rag2 KO mouse model. Tumor kinetics after s.c. injection with 2 × 10^6 SCC22b-S mixed with 25% Matrigel (n = 5). Each line represents the data of one individual mouse. HNSCC: Head and neck squamous cell carcinoma; KO: knock-out; -S: cetuximab-sensitive HNSCC cell line; s.c.: subcutaneous.

without 25% Matrigel, at a concentration of 1 × 10^6 cells per injection. CB17 Scid mice are genetically engineered to have no T and B cells, but still have intact innate immunity, including NK cells. The BALB/c nude mice lack T cells, but not B cells. In addition, NK cells are present at normal levels, and therefore, this model is also suitable to investigate NK cell cytotoxic responses such as ADCC. For this pilot experiment, we chose to only evaluate the resistant variants of our cell lines.

Only two out of three CB17 Scid mice injected with 1 × 10^6 SC263-R cells developed tumors, although very late in the experimental period, i.e., on days 31 and 45 post inoculation [Figure 4A]. In contrast, none of the BALB/c nude mice injected with only tumor cells showed any tumor growth [Figure 4B]. Interestingly, all CB17 Scid mice injected with 1 × 10^6 tumor cells mixed with 25% Matrigel exhibited sustainable tumor growth, which was already measurable as early as day 5 post inoculation. These mice reached their endpoint (tumor size = 150 mm^2) on days 61 and 91 [Figure 4C]. While two out of three BALB/c nude mice injected with tumor cells mixed with 25% Matrigel also developed tumors, growth was not sustained in this mouse strain [Figure 4D]. To maximize the information obtained from our pilot experiment and gain insight into the ability of SCC22b-R cells to grow in different mouse strains, mice that did not exhibit any tumor growth after injection with SC263-R cells were subsequently injected with SCC22b-R cells with Matrigel (for CB17 Scid mice) and with/without Matrigel (for BALB/c nude mice) at the opposite flank. However, none of the mice demonstrated sustainable tumor growth over time (data not shown). In conclusion, our pilot experiment suggested that the CB17 Scid mouse strain is an appropriate model for xenografting the human HNSCC SC263-R cell line in combination with Matrigel co-injection.

Resistant SC263-R cells do not maintain their cetuximab resistance in CB17 Scid mice

Now that we have identified an appropriate mouse model, we proceeded to the next phase of our study, which involved testing the effectiveness of cetuximab. Dose titration of the EGFR-targeting mAb cetuximab was performed in order to investigate whether xenografted HNSCC cells retained their cetuximab sensitivity in an *in vivo* setting. As tumor growth was rather slow, we increased the number of HNSCC cells to be injected from 1 × 10^6 to 2.5 × 10^6 with 25% Matrigel. CB17 Scid mice were injected with either SC263-S or SC263-R cells to generate a model that is sensitive and resistant to cetuximab, respectively. When tumors reached a volume of approximately 30 mm^2, mice were randomized into four treatment groups (vehicle, cetuximab low dose, cetuximab medium dose, and cetuximab high dose). Unfortunately, mice inoculated

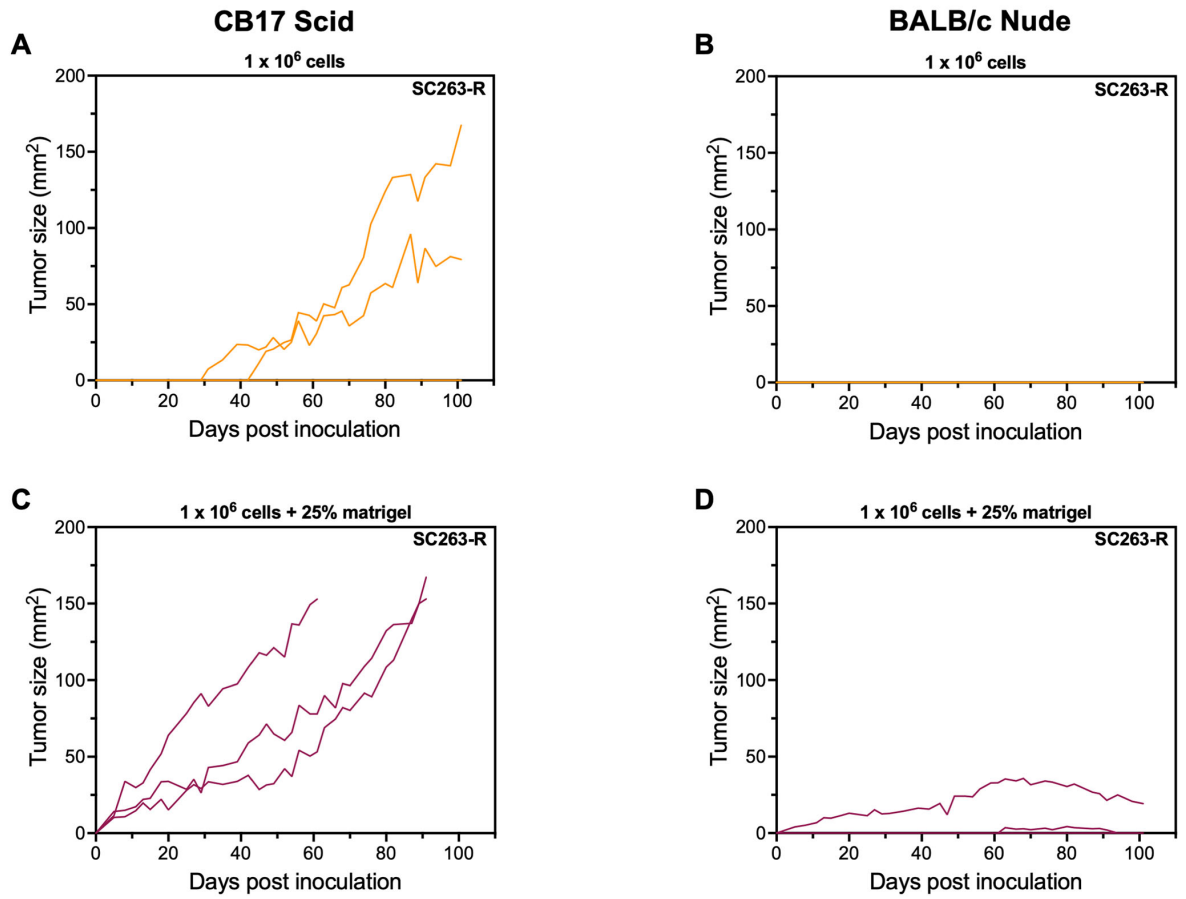

Figure 4. CB17 Scid mice are suitable hosts for SC263-R HNSCC cells when co-injected with 25% Matrigel. (A and B) Tumor kinetics over time after s.c. injection with 1 × 10^6 SC263-R cells in CB17 Scid: (A) and BALB/c nude; (B) mice (n = 3); (C and D) Tumor kinetics over time after s.c. injection with 1 × 10^6 SC263-R cells mixed with 25% Matrigel in CB17 Scid: (C) and BALB/c nude; (D) mice (n = 3). Each line represents the data of one individual mouse. HNSCC: Head and neck squamous cell carcinoma; -R: cetuximab-resistant HNSCC cell line; -S: cetuximab-sensitive HNSCC cell line; s.c.: subcutaneous.

with the SC263-S cell line reached an average tumor size of a maximum of 25 mm^2, after which the tumors spontaneously started to decrease in size for unknown reasons prior to treatment [Figure 5A]. In a final attempt to induce sustainable tumor growth of the SC263-S cell line in CB17 Scid mice, we remarkably increased the cell number and injected the mice with 10 × 10^6 SC263-S cells. Due to a global shortage of Matrigel, we used 12 mg/mL Geltrex instead to promote tumor growth. Geltrex has successfully been used in our lab to grow solid tumors from hematological cancer cell lines and is therefore a good alternative for Matrigel. Unfortunately, the results were similar to the previous experiment using 2.5 × 10^6 injected cells in CB17 Scid mice. Despite the tumors initially growing up to a maximum size of approximately 60 mm^2, they eventually began to regress spontaneously [Figure 5B]. Hence, while the tumors in this experiment achieved a larger size than in the previous one, we were still unable to induce durable tumor growth.

In contrast, in the mice injected with the SC263-R cells, treatment could be initiated at day 7. Mice in the vehicle group showed linear tumor growth, whereas tumors in the treatment groups started to decrease from the moment of treatment initiation. This shrinkage of tumor volume in the treatment groups continued until the mice showed no visible/palpable tumor anymore [Figure 5C].

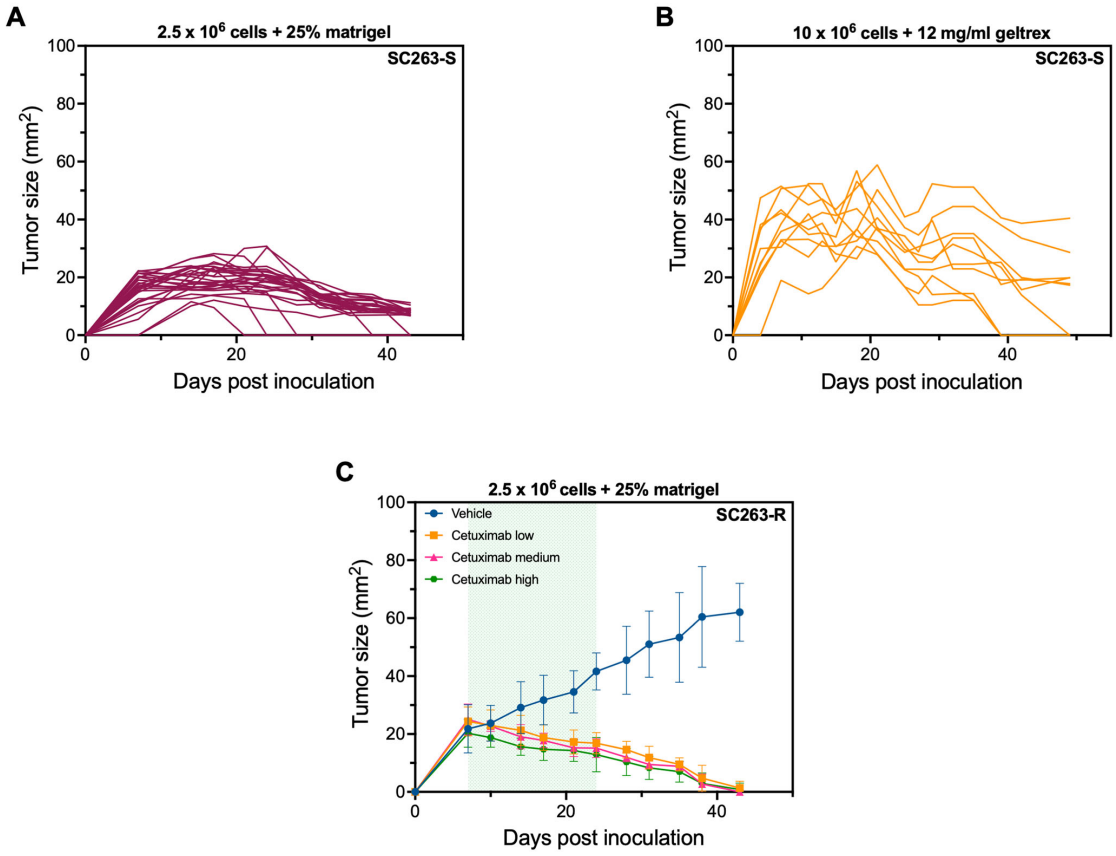

Figure 5. SC263 xenograft models in CB17 Scid mice are unsuitable for investigating cetuximab resistance. (A) Tumor kinetics after s.c. injection with 2.5 × 10^6 + 25% Matrigel SC263-S cells in CB17 Scid mice (n = 27); (B) Tumor kinetics after s.c. injection with 10 × 10^6 + 12 mg/mL Geltrex SC263-S cells in CB17 Scid mice (n = 10). Each line represents the data of one individual mouse; (C) Tumor kinetics of SC263-R tumor-bearing CB17 Scid mice following treatment with vehicle (PBS, n = 6), cetuximab low (2.5 mg/kg, n = 7), cetuximab medium (10 mg/kg, n = 7), and cetuximab high (50 mg/kg, n = 7). The green area in the graph represents the treatment period (starting from day 7). Data represent mean ± SD. HNSCC: Head and neck squamous cell carcinoma; -S: cetuximab-sensitive HNSCC cell line; -R: cetuximab-resistant HNSCC cell line; s.c.: subcutaneous.

Immunohistochemistry demonstrates the high presence of macrophages in SC263-R tumors induced in CB17 Scid mice

As the CB17 Scid mouse strain has an intact innate immune system, the above-mentioned results might be explained by an activation of innate immune cells. To investigate this, we harvested tumors from both SC263-S and SC263-R tumor-bearing mice and performed immunohistochemistry [Figure 6 and Supplementary Figure 1]. Untreated SC263-S tumors were characterized by a low to moderate proliferation rate, little to no apoptotic cells, a moderate abundance of macrophages, and little to no NK cells. In contrast, Ki67 staining in vehicle-treated SC263-R-tumor-bearing mice indicated a high proliferation rate in these tumors. However, cetuximab treatment resulted in lower Ki67+ cells, indicating that cetuximab was inhibiting tumor cell proliferation in resistant cells. Although higher compared to SC263-S tumors, cleaved caspase-3 staining in SC263-R tumors was limited and slightly increased in the treatment groups. F4/80+ macrophages unexpectedly infiltrated the cetuximab-treated SC263-R tumors (particularly in the cetuximab low treatment group). This suggests that cetuximab may be altering the tumor microenvironment by increasing the infiltration of macrophages into the tumor, causing the *in vitro* resistant cells to lose their resistance *in vivo*. Little to no NKp46+ cells were present in the vehicle group, but when tumors were treated with cetuximab, more NK cells appeared. However, this increase was not as pronounced as the infiltration

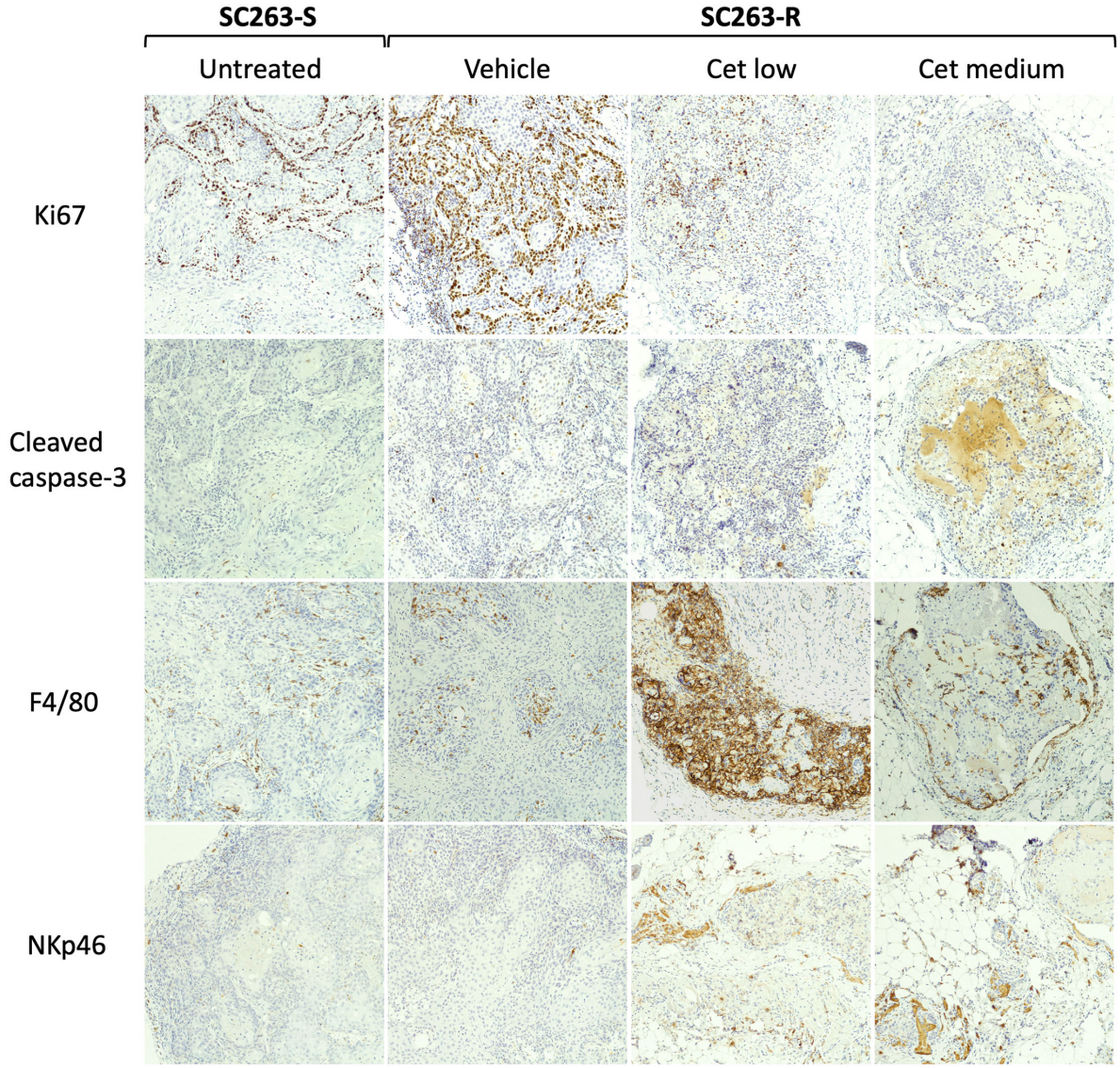

Figure 6. Infiltration of macrophages is likely compromising the growth of SC263-S tumors and the resistance to cetuximab of SC263-R tumors in CB17 Scid mice. Representative images of immunohistochemical staining for Ki67 (proliferation), cleaved caspase-3 (apoptosis), F4/80 (macrophages), and NKp46 (NK cells), shown at 100x. Vehicle: PBS; Cet low: 2.5 mg/kg cetuximab; Cet medium: 10 mg/kg cetuximab. NK: Natural killer; PBS: phosphate-buffered saline; -R: cetuximab-resistant HNSCC cell line; -S: cetuximab-sensitive HNSCC cell line.

of macrophages observed in treatment groups. Overall, these results suggest that there is a predominant presence of macrophages in both SC263-S and SC263-R tumor-bearing mice, which may be impairing the growth of SC263-S cells and the resistance of SC263-R to cetuximab *in vivo*.

FaDu cell lines retain their *in vitro* sensitivity status to cetuximab in CB17 Scid mice

In a final attempt to establish a reliable *in vivo* model for cetuximab resistance, we changed the HNSCC cell lines from SC263 to FaDu (sensitive and acquired resistant variant). In a pilot experiment with these cell lines in CB17 Scid mice, both the FaDu-S and FaDu-R cell lines exhibited robust tumor growth without the

need to add Matrigel or Geltrex [Supplementary Figure 2A and B]. This stands in contrast to the SC263-S cell line, which failed to demonstrate sustainable tumor growth in this mouse model [Figure 5A and B]. To investigate whether FaDu cells maintained their *in vitro* resistance status to cetuximab, mice were injected with 1×10^6 FaDu-S or FaDu-R cells. When tumors reached a size of approximately 30 mm^2, mice were randomized and treated with vehicle or a low dose of cetuximab (2.5 mg/kg). We opted to test only a low dose of cetuximab, since tumors already completely disappeared with this dosage in SC263-R tumor-bearing CB17 Scid mice [Figure 5C]. Treatment was initiated on days 13 and 9 post-inoculation for mice with FaDu-S and FaDu-R tumors, respectively. Treatment with cetuximab resulted in a significant delay in tumor growth in FaDu-S-bearing mice, while FaDu-R-bearing mice demonstrated persistent tumor growth upon cetuximab treatment [Figure 7A and B, Supplementary Figure 2C and D].

Since tumor size has been reported to be inversely correlated to response to EGFR inhibitors and chemotherapy[43-45] in patients and CBA/lac mice, we next investigated the response to cetuximab in more established tumors in both models by delaying treatment initiation to a tumor size of approximately 70 mm^2. In these more established models, FaDu-S and FaDu-R tumor-bearing mice reached the treatment initiation point on day 18 post inoculation. Cetuximab treatment effectively reduced tumor growth exclusively in the FaDu-S tumor-bearing mice [Figure 7C and D], indicating that even in more established and advanced tumors, cetuximab resistance status is maintained. However, due to the study's implemented humane endpoints, the treatment window was too small to complete the intended three-week treatment period. To obtain more insight into the tumor and its microenvironment, immunohistochemistry was performed and demonstrated a remarkable decrease in Ki67+ cells and a slight increase in macrophages after cetuximab treatment in these tumors [Figure 7E]. The former confirms that cetuximab effectively reduced tumor proliferation in cetuximab-sensitive FaDu-S tumors but not in resistant FaDu-R tumors. Cetuximab treatment had no effect on the level of apoptosis or NK cell infiltration [Supplementary Figure 2E]. In conclusion, the FaDu-S and FaDu-R cell lines effectively maintain their *in vitro* resistance status to cetuximab *in vivo* in CB17 Scid mice, even when tumors are more established. As such, these robust and reliable models underscore the suitability and promise of using xenografts with both sensitive and acquired resistant FaDu HNSCC cell lines for investigating novel combination therapies aimed at overcoming cetuximab resistance in an *in vivo* setting.

DISCUSSION

Cetuximab resistance poses a significant challenge in the search for effective treatment options for HNSCC. To understand and address this problem, it is crucial to develop *in vivo* models that accurately depict cetuximab resistance. Such clinically relevant animal models are essential for studying and exploring novel combinations that have the potential to overcome resistance to cetuximab. Over the years, xenografts have become the gold standard for investigating novel cancer treatments, as they allow the use of human cell lines or even patient samples[46]. However, the majority of studies addressing novel cancer treatments in HNSCC have primarily relied on xenograft models using human cell lines, without specifically focusing on resistance nor on retaining intact ADCC functionality *in vivo*. Studies specifically using an acquired cetuximab-resistant xenograft model in HNSCC are rather scarce and appear to be challenging to develop[47,48]. As far as our knowledge extends, our research represents the first successful establishment of an acquired cetuximab-resistant HNSCC model derived from *in vitro*-generated acquired resistant cells. In addition to our work, there have been previous attempts to establish *in vivo* models of acquired cetuximab resistance by chronically treating tumor-bearing mice with increasing doses of cetuximab, however, without success for HNSCC[47,48]. There has also been a study that established an HNSCC *in vivo* mouse model by utilizing cells with the EGFR-K521 polymorphism that are intrinsically resistant to cetuximab[49]. However, our work specifically focuses on acquired resistance, making our study distinct and unique in the field. In

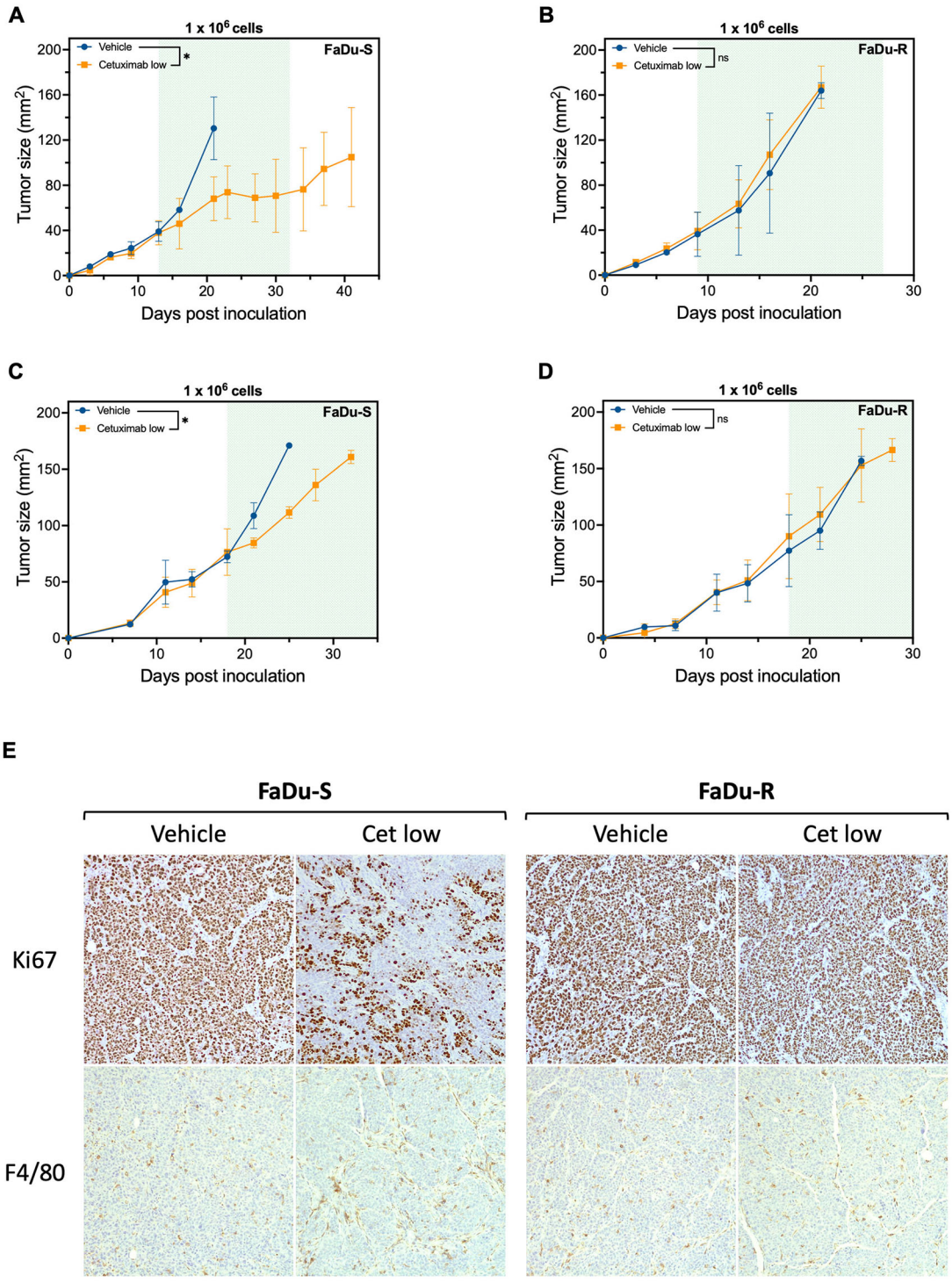

Figure 7. Cetuximab resistance status of isogenic FaDu cell lines is maintained in CB17 Scid mice. (A-D) Tumor kinetics of CB17 Scid mice inoculated with 1 × 10^6 cetuximab sensitive FaDu-S cells (A and C) or cetuximab resistant FaDu-R cells (B and D) following treatment with vehicle (PBS, *n* = 2) or cetuximab low (2.5 mg/kg, *n* = 3). The green area in each graph represents the treatment period [starting from (A) day 13, (B) day 9 or (C and D) day 18]. Data represent mean ± SD. *P*-values were determined using a linear mixed model; (E) Representative images of immunohistochemical staining for Ki67 (proliferation) and F4/80 (macrophages), shown at 100x. **P* < 0.05. ns: Non-significant; PBS: phosphate-buffered saline; -R: cetuximab-resistant HNSCC cell line; -S: cetuximab-sensitive HNSCC cell line.

contrast, more success has been achieved with patient-derived xenograft mouse models for acquired cetuximab resistance, offering a valuable alternative and clinically relevant approach to study acquired cetuximab resistance *in vivo*[50,51].

In the present study, we aimed to establish a xenograft model of acquired resistance using a human HNSCC cell line that was initially made resistant *in vitro* by chronically exposing it to increasing doses of cetuximab[7]. Our attempts to generate a resistance model using the Rag2 KO mouse strain were unsuccessful, despite various attempts to optimize tumor growth induction by adjusting tumor cell number, cell lines and/or the addition of Matrigel. The finding that not even the SCC22b-S, which has only a larger passage number than its parental cell line, failed to induce sustainable tumor growth is particularly surprising, considering the extensive use of the parental cell line in numerous published xenograft studies[39-42], yet in other mouse models than Rag2 KO and not focusing on cetuximab resistance. Initially, we chose the Rag2 KO mouse model, since this strain has been reported as a suitable host for tumor implantation studies of several cancers, including HNSCC[52-55]. This is because the loss of Rag2 blocks the development of mature B and T cells, creating an immunodeficient mouse model that should be able to accept allogenic transplants[55-57]. The C57BL/6N genetic background of the mouse strain employed in our study could potentially explain the unsuccessful tumor engraftment. C57BL/6N mice have a more robust and aggressive innate immunity in comparison to mice with a BALB/c background[58-62]. This more robust immune activity could have triggered an amplified immune response against the implanted tumor cells, ultimately inhibiting their growth and engraftment in the Rag2 KO mouse strain.

Next, we conducted a pilot experiment using CB17 Scid and BALB/c Nude mice, two different mouse strains on a BALB/c genetic background with intact innate immunity that have been extensively used in HNSCC xenograft studies[37,41,63-65], although not specifically in the context of cetuximab. Based on this experiment, the CB17 Scid mouse strain was selected as a suitable model for xenografting the SC263-R cell line. It is important to note that a limitation of this pilot experiment was the exclusion of the sensitive cell line (SC263-S), primarily due to the limited availability of mice, which was later found to be unable to grow in this specific mouse strain. Additionally, we observed that the acquired cetuximab-resistant variant SC263-R had lost its resistance phenotype in this specific *in vivo* model. This observation has been reported in literature before, although to a limited extent. In this regard, the study of Formelli *et al.* showed that doxorubicin-resistant B16 melanoma cells only maintained their resistance *in vivo* when the *in vitro* resistance index was greater than 100[66]. Similarly, when the *in vitro*-derived cetuximab-resistant SCC1c8 HNSCC cell line was transplanted into an athymic nude mouse model, it unexpectedly lost its resistance phenotype[48]. This mouse model lacks T cells but has functional B cells, NK cells and macrophages[67], which may have been the reason for the observed loss of resistance *in vivo*. Similarly, an *in vitro* generated breast cancer cell line resistant to (ADCC-capable) trastuzumab failed to maintain its resistance status in an *in vivo* mouse model[68]. These outcomes highlight the limitations of solely relying on *in vitro* drug exposure to generate resistant clones. To ensure more reliable *in vivo* models of drug resistance, it is suggested that *in vivo* selection or a combination of *in vivo* and *in vitro* selection methods should be employed[69]. However, *in vivo* selection is still challenging and does not guarantee the successful generation of resistance models. This was demonstrated by Quesnelle *et al.*, who performed an *in vivo* selection of 10 different HNSCC cell lines, including SCC22b, with the goal of establishing much-needed *in vivo* models of cetuximab resistance. However, despite their efforts, none of the cell lines exhibited successful acquisition of cetuximab resistance in their *in vivo* setting[47]. In addition, *in vivo* generated cetuximab-resistant cancer cells demonstrated to slowly lose their resistance phenotype after several *in vitro* passages[70,71]. The cetuximab-resistant cell lines used in the present study were solely generated *in vitro*, but have proven to maintain their resistance phenotype even after 6 weeks of culture without cetuximab[7,72], excluding the latter as a possible reason for the loss of resistance *in vivo*.

As immunohistochemical analysis revealed a high presence of macrophages in both SC263-S and SC263-R tumors, these macrophages might have impaired the growth of SC263-S cells and the resistance phenotype of SC263-R cells. The presence of macrophages in the tumor microenvironment may have exerted suppressive effects on tumor growth by contributing to an antitumor immune response or by directly influencing tumor cell behavior. Nevertheless, we acknowledge that our immunohistochemical findings warrant further validation with larger sample sizes to ensure robustness and reliability before drawing any definitive conclusions. In addition, factors such as oxygen levels, nutrient availability, cell-cell interactions and the presence of stromal cells, which are all different or even absent in *in vitro* cell cultures, may influence the behavior of tumor cells and potentially impact their response to cetuximab. In this regard, it has been shown that hepatocellular cancer cells cultivated *in vitro* in more native conditions exhibited an altered drug sensitivity compared to cells cultured in standard conditions[73], highlighting the influence of the tumor microenvironment on drug sensitivity[74].

In a final attempt to establish a cetuximab-resistant *in vivo* model from an *in vitro* generated resistant HNSCC cell line, we utilized acquired resistant FaDu-R cells. The parental FaDu cell line has already been used in HNSCC xenograft studies[64,75-77], and both FaDu-S and FaDu-R have been demonstrated to induce robust tumor growth in CB17 Scid mice. Moreover, we showed that both FaDu-S and FaDu-R cell lines maintain their sensitivity/resistance status to cetuximab *in vivo*, in contrast to our other *in vitro*-proven cetuximab-resistant HNSCC cell lines.

Previous studies in mice have demonstrated that more advanced tumors have a lower response to EGFR inhibitors and chemotherapy[43,44]. Together with the fact that larger gross tumor volumes have been linked to worse outcomes in HNSCC patients receiving cetuximab and radiotherapy[73], we delayed treatment initiation until the tumor was more established, as it might lead to reduced responsiveness to cetuximab. Yet, FaDu-S and FaDu-R cells maintained their resistance phenotype when treatment was initiated at a larger tumor size. However, they exhibited rapid growth, failing to complete the intended three-week treatment period. Overall, this demonstrates that we have succeeded in establishing reliable and robust HNSCC mouse models, where the cetuximab resistance status of the tumor cells remains unaffected by larger tumor sizes.

It is important to mention that our models for cetuximab resistance have certain limitations. More specifically, we established tumor models using both cetuximab-sensitive and -resistant variants derived from only one HPV-negative cell line of hypopharyngeal origin, restricting our models to fully capture the heterogeneity and complexity of the HNSCC patient population. Indeed, one single HNSCC cell line may not fully capture the diverse molecular and phenotypic profiles observed in different patients. The choice to focus only on HPV-negative HNSCC can be justified by the fact that this patient population is in greater need of novel treatment options, as they have an inferior prognosis in terms of recurrence and survival compared to HPV-positive patients[78]. In addition, HPV-positive HNSCC patients are, in the majority of cases, intrinsically resistant to cetuximab[79,80], making an acquired cetuximab-resistant *in vivo* model for this patient population less clinically relevant. It is also worth mentioning that our established models are only validated for low doses (2.5 mg/kg) of cetuximab with a treatment period of three weeks. Further validation of the models with higher doses is still required. Furthermore, since our models are xenograft models, these mice lack a fully functional immune system, specifically the adaptive immunity component, which plays a crucial role in the evaluation of immunotherapeutic agents, such as pembrolizumab and nivolumab[81]. However, previous studies in literature have utilized similar mouse strains with only ADCC induction

capability and no adaptive immunity to evaluate cetuximab-containing treatment combinations[31,32,40,82-84], highlighting these models as valuable research tools. Although a syngeneic model would address the limitation of lacking adaptive immunity in our CB17 Scid mouse models, its use was not considered suitable for our study due to the inability of cetuximab to bind to murine EGFR[85]. While there is a mouse variant of cetuximab known as 7A7, which was initially proposed as a valuable antibody for EGFR-based preclinical studies in mice[86], a recent study failed to reproduce the earlier reported results[87]. Furthermore, apart from the laboratory that initially reported 7A7, there are no other published studies in literature that have used this specific antibody, despite the first report dating back 20 years. Considering these factors, we opted against using a syngeneic model for our study, as it would not have provided the necessary compatibility with cetuximab and an accurate representation of its effects in mice. Alternatively, humanized mouse models could be a more optimal choice, as they possess a complete human immune system, including human NK cells[81]. However, humanized mouse models can be costly to establish and maintain, making them a financial challenge for many research laboratories. Therefore, our models consider a robust and economical approach for cetuximab resistance and combination studies *in vivo*.

In conclusion, we have successfully established *in vivo* mouse models for cetuximab resistance and sensitivity using the FaDu-R and FaDu-S cell lines, respectively, in CB17 Scid mice with intact ADCC functionality. These models provide a useful tool for studying resistance mechanisms and novel drug combination strategies in a more clinically relevant setting.

DECLARATIONS

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All animal care and experimental procedures were approved by the Ethics Committee for Animal Experiments of the University of Antwerp (2020-41 and 2021-39).

Consent for publication

Not applicable.

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Review

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Recent advances in natural compounds inducing non-apoptotic cell death for anticancer drug resistance

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Abstract

The induction of cell death is recognized as a potent strategy for cancer treatment. Apoptosis is an extensively studied form of cell death, and multiple anticancer drugs exert their therapeutic effects by inducing it. Nonetheless, apoptosis evasion is a hallmark of cancer, rendering cancer cells resistant to chemotherapy drugs. Consequently, there is a growing interest in exploring novel non-apoptotic forms of cell death, such as ferroptosis, necroptosis, pyroptosis, and paraptosis. Natural compounds with anticancer properties have garnered significant attention due to their advantages, including a reduced risk of drug resistance. Over the past two decades, numerous natural compounds have been discovered to exert anticancer and anti-resistance effects by triggering these four non-apoptotic cell death mechanisms. This review primarily focuses on these four non-apoptotic cell death mechanisms and their recent advancements in overcoming drug resistance in cancer treatment. Meanwhile, it highlights the role of natural compounds in effectively addressing cancer drug resistance through the induction of these forms of non-apoptotic cell death.

Keywords: Drug resistance, cancer therapy, natural compound, non-apoptotic cell death

INTRODUCTION

Cancer, as a major global public health concern, poses a severe threat to people's well-being. In 2018, the World Health Organization reported that cancer ranked the second leading cause of death globally, resulting in approximately 9.6 million annual fatalities[1]. Cell death is a fundamental process crucial for human health, playing a pivotal role in regulating cell division, facilitating organ development, and upholding tissue homeostasis[2]. Nevertheless, the disruption and avoidance of cell death mechanisms facilitate malignant cell transformation and advance tumorigenesis[3]. From a therapeutic perspective, conventional cancer treatments, such as chemotherapy and radiation, achieve their anticancer effects by inducing cell death[2].

Over the span of several decades, there has been a growing comprehension of the diverse mechanisms governing cell death. This process can be classified into two primary categories: programmed cell death (PCD), which is genetically controlled, and unprogrammed cell death, which represents a passive response to both biotic and abiotic stress. Among the various forms of PCD, apoptosis has emerged as one of the earliest and most extensively explored pathways. For a significant duration, the development of anticancer drugs targeting apoptosis has been a focal point of research. Numerous drugs, such as cisplatin, oxaliplatin, and pirarubicin, have showcased their ability to elicit anticancer effects through this mechanism[4,5]. However, recent studies have increasingly validated that cancer cells possess the capability to elude apoptosis through a range of mechanisms, including the overexpression of apoptosis-inhibiting proteins, the inhibition of apoptosis-inducing factors, and the activation of survival signaling pathways[6]. This evasion of apoptosis directly contributes to chemoresistance, a phenomenon intricately associated with alterations in various aspects of cancer, encompassing angiogenesis, the tumor microenvironment, and oxidative stress[7-10]. Ultimately, this leads to the failure of cancer treatment. To improve the efficacy of cancer treatment, the exploration of non-apoptotic cell death mechanisms has gradually emerged as a prominent priority. Over the last two decades, researchers have sequentially unveiled and extensively investigated novel different cell death mechanisms, including ferroptosis, necroptosis, pyroptosis, and paraptosis. These unique mechanisms, each with its distinct regulators and pathways, hold the potential to be activated within apoptosis-resistant cancer cells, offering novel strategies for the treatment of cancer and overcoming cancer drug resistance.

Throughout history, natural compounds derived from plants, animals, microorganisms, and minerals have consistently served as a valuable source for drug discovery[11,12]. Unlike synthetic compounds, these substances are not artificially created and can be categorized into various forms, such as alkaloids, flavonoids, and terpenes, due to their diverse chemical structures[13]. Many of these natural compounds exhibit significant potential in the realm of cancer treatment. In fact, certain natural compounds, such as paclitaxel (PTX), camptothecin, and vincristine, have already gained widespread acceptance as chemotherapeutic drugs in clinical practice[14,15]. Moreover, an interesting aspect is that numerous natural compounds have been found to induce various non-apoptotic cell death pathways when administered to resistant cancer cells that evade apoptosis.

This review focuses on ferroptosis, necroptosis, pyroptosis, and paraptosis, providing a comprehensive overview of the latest research advancements in these mechanisms within the framework of cancer. We place specific emphasis on their relevance in the context of combatting cancer drug resistance. Additionally, this review compiles information on natural compounds with the capacity to induce these four modes of cell death in the context of addressing cancer resistance over the past two decades.

FERROPTOSIS

Overview of ferroptosis

Ferroptosis, a recently discovered form of PCD that is iron-dependent, was first proposed in 2012[16]. Morphologically, ferroptotic cells exhibit intact nuclei without chromatin condensation. However, their mitochondria undergo significant changes, including reduced size, increased membrane density, reduced cristae, and outer membrane rupture[17]. Interestingly, the discovery of ferroptosis inducers was earlier than its naming. Yang *et al.* discovered some new compounds as early as 2003 and 2008, including Erastin, RSL3, and RSL5, which induce cell death through a mechanism distinct from apoptosis[18]. Erastin reduces cysteine uptake by inhibiting cysteine/glutamate transporter receptor, known as System X_c^-, resulting in a decrease in glutathione (GSH) synthesis and an increase in iron-dependent lipid peroxidation (LPO), which finally leads cells to ferroptosis[19]. Differently, RSL3 directly inhibits the activity of Glutathione Peroxidase 4 (GPX4), a GSH-utilizing enzyme that prevents the accumulation of toxic lipid hydroperoxide, thereby inducing ferroptosis[20]. Additionally, Erastin also indirectly triggers ferroptosis through the inactivation of GPX4 via the inhibition of GSH synthesis[21].

It is known that LPO and intracellular iron accumulation play pivotal roles in triggering ferroptosis. Polyunsaturated fatty acids (PUFAs) are the most susceptible lipids to peroxidation during ferroptosis. The regulation of PUFA synthesis may be influenced by enzymes such as Long-chain acyl-CoA synthetases (ACSLs) and lyso-phosphatidylcholine acyltransferase-3 (LPCAT3). Additionally, arachidonate lipoxygenases (ALOXs) and cytochrome p450s (POR) can directly or indirectly modulate PUFA peroxidation, resulting in ferroptosis[22]. Additionally, iron metabolism is crucial for ferroptosis. As Fe^{2+} is released from the labile iron pool (LIP) into the cytoplasm, excess Fe^{2+} oxidizes PUFAs to hydroxyl radicals and leads to ferroptosis.

The mitochondria are responsible for cellular metabolism and also play an important role in the regulation of ferroptosis[23]. Within the mitochondria, dihydroorotate dehydrogenase (DHODH) reduces ubiquinone (CoQ) to ubiquinol (CoQH2), serving as a radical-trapping antioxidant with anti-ferroptosis activity. DHODH plays a significant role in mediating ferroptosis defense independent of the GSH pathway[24]. Similarly, the plasma enzyme ferroptosis suppressor protein 1 (FSP1) inhibits lipid hydroperoxides by reducing ubiquinone to ubiquinol, operating in parallel with GPX4 to counteract ferroptosis[25]. Obviously, the mechanisms of ferroptosis are complex and being explored constantly [Figure 1].

Ferroptosis pathways for chemotherapy resistance in cancer

In recent years, ferroptosis has been a hot topic in cancer development, treatment and cancer drug resistance. Several signaling pathways have been found to participate in promoting cancer development and drug resistance through the inhibition of ferroptosis. With multiple functions for proliferation, metastasis, and differentiation of cancer cells, the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of the rapamycin (mTOR) signaling pathway is responsible for ferroptosis prevention via sterol regulatory element-binding protein 1 (SREBP1)/stearoyl-CoA desaturase-1 (SCD1)-mediated adipogenesis[26]. Hippo pathway activity is also responsible for cell growth and proliferation. Activating the Hippo pathway can suppress downstream YAP, leading cancer cells resistant to ferroptosis by downregulating acyl-CoA synthetase long-chain family member 4 (ACSL4) and TCP friendly rate control (TFRC)[27]. Since RAS may regulate some processes to escape ferroptosis, RAS-mutated cells are always susceptible to ferroptosis. High-mobility group box 1 (HMGB1), a leukemia pathogenic gene, inhibits LPO via the RAS/MAP kinase (MAPK) pathway, promoting ferroptosis resistance[28].

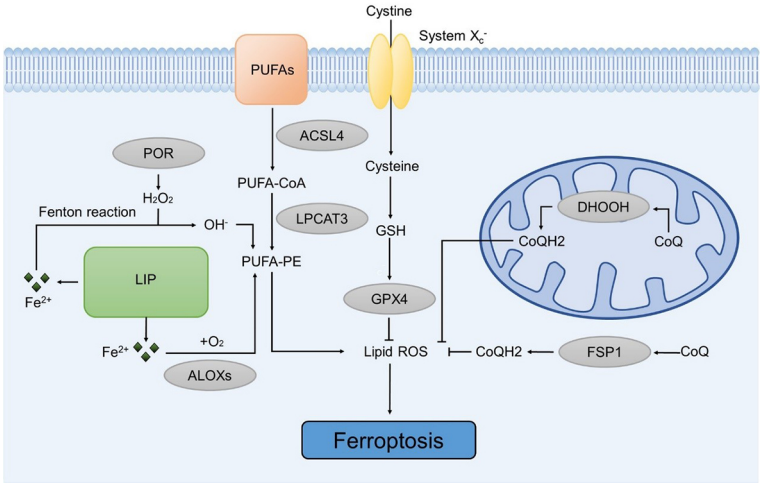


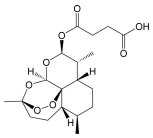
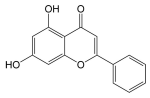
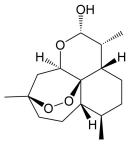
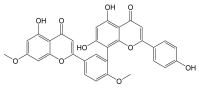
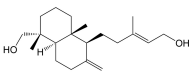
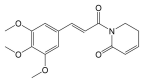
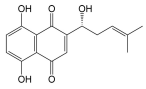
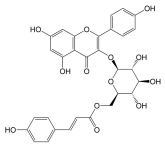
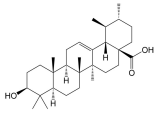
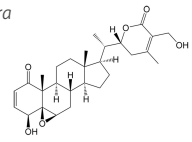
Figure 1. The mechanism of ferroptosis. Ferroptosis can be initiated by an increase in intracellular iron levels and the accumulation of iron-dependent lipid peroxidation. It can be induced through the SystemX_c^-/GSH/GPX4 axis, the DHODH/CoQ10 axis, and the FSP1/CoQ10 axis. ACSL: Acyl-CoA synthetase; ALOXs: arachidonate lipoxygenases; CoQ: ubiquinone; FSP1: ferroptosis suppressor protein 1; GPX4: glutathione peroxidase 4; GSH: glutathione; LPCAT3: lyso-phosphatidylcholine acyltransferase-3; LIP: labile iron pool; POR: cytochrome p450s; PUFA: polyunsaturated fatty acid.

Additionally, several other pathways, including nuclear factor erythroid 2-related factor 2 (Nrf2), p53, and hypoxia-inducible factor (HIF), are also involved in chemotherapeutic resistance through ferroptosis regulation. Recent research highlights the significant impact of the Nrf2 signaling pathway on both organ protection and resistance to cisplatin (DDP) across various cancer types[29]. For instance, in non-small cell lung cancer, Erastin and Sorafenib, either alone or in combination, induce ferroptosis by inhibiting the Nrf2/SLC7A11 (also known as xCT) pathway, thereby overcoming DDP resistance[30]. The p53 gene, extensively studied in cancer research, influences metabolic pathways related to ferroptosis, such as enhancing ferroptosis by down-regulating xCT expression[31]. According to this mechanism, Flubendazole and 5-fluorouracil (5-FU) demonstrate synergistic effects in treating castration-resistant prostate cancer[32]. HIF serves a dual role in the regulation of ferroptosis in cancer cells. Lowering HIF-α levels has been found to increase LPO and enhance ferroptosis in clear cell renal cell carcinoma[33]. Conversely, in temozolomide-resistant glioblastoma, activating HIF-1α and HIF-2α can induce ferroptosis, with HIF-2α possibly promoting LPO as the primary mechanism[34].

Natural compounds inducing ferroptosis for cancer treatment

Many natural compounds have been found to induce ferroptosis via single or combinational therapies, offering opportunities for cancer treatment and drug resistance [Table 1]. Here, we summarize those natural compounds that reverse drug resistance by inducing ferroptosis. Most of these compounds can synergize with chemotherapeutic drugs by regulating ferroptosis-related proteins and genes. Recent studies have revealed that sorafenib can induce ferroptosis in various types of cancer[35]. However, sorafenib-induced ferroptosis can be suppressed, specifically through the activation of the PI3K/Akt signaling pathway or the NRF2/GPX4 axis[36,37]. To overcome this challenge, drug combinations have proven to be effective strategies. Dihydroartemisinin, a derivative of artemisinin, shares similar mechanisms with sorafenib regarding ferroptosis-related proteins, such as GPX4, and has a stronger effect in liver cancer cells when combined with sorafenib[38]. Ursolic acid, a pentacyclic triterpene compound, also exhibits synergistic anticancer effects with sorafenib by inhibiting xCT in many cancers[39]. While DDP is considered a front-line chemotherapy drug for various cancers, including ovarian cancer, its resistance poses a significant impediment to achieving effective treatment outcomes[40]. Inhibition of apoptosis plays a significant role in

Table 1. Natural compounds for anticancer drug resistance by inducing ferroptosis

Compounds	Origin	Structure	Cancer	Anti-drug resistant effects	Refs
Artesunate	Artemisia annua L.		Renal cell carcinoma	Increasing cytotoxicity in sunitinib-resistant renal cell carcinoma by triggering ferroptosis, increasing ROS generation, and decreasing metabolism.	[50]
Chrysin	Oroxylum indicum (L.) Kurz		Pancreatic cancer	Inhibiting CBR1 activity in gemcitabine-resistant pancreatic cancer to trigger ferroptosis through ROS accumulation.	[48]
Dihydroartemisinin	Artemisia annua L.		Hepatocellular carcinoma	Synergizing with sorafenib to induce ferroptosis by increasing the levels of L-ROS, LIP, and MDA and decreasing the level of GSH.	[38, 127-129]
Ginkgetin	Ginkgo biloba L.		Non-small cell lung cancer	Synergizing with DDP to induce ferroptosis by increasing ROS formation, decreasing the expression of xCT and GPX4, and inactivating the Nrf2/HO-1 axis.	[46]
Kayadiol	*Torreya nucifera* Sieb. et Zucc. (Taxaceae)		Extranodal natural killer/T cell lymphoma	Inducing p53-mediated ferroptosis through the xCT/GPX4 axis and exhibiting synergistic effects when combined with L-asparaginase and DDP.	[49]
Piperlongumine	*Piper longum* L.		Pancreatic cancer	Enhancing the antitumor effects of erastin by inducing ROS generation, GSH depletion and inhibiting TXNRD activity.	[43, 130]
Shikonin	*Lithospermum erythrorhizon* Sieb. et Zucc.		Ovarian cancer	Synergizing with DDP to induce ferroptosis through upregulation of HMOX1 and increased levels of ROS, LPO, and Fe^{2+}.	[42]
Tiliroside	*Tribulus terrestris* L.		Hepatocellular carcinoma	Enhancing the antitumor effects of sorafenib by inducing ferroptosis via targeting TBK1 to promote Keap1-mediated Nrf2 ubiquitination and degradation.	[45]
Ursolic acid	*Ligustrum lucidum* W. T. Aiton		Colon cancer, gastric cancer, prostate cancer	Enhancing the antitumor effects of sorafenib by inducing xCT-dependent ferroptosis.	[39]
Withaferin A	*Withania somnifera* (L.) Dunal		Hepatocellular carcinoma	Enhancing the antitumor effects of sorafenib in sorafenib-resistant hepatocellular carcinoma cells by regulating Keap1/Nrf2-associated ferroptosis and EMT.	[44]

CBR1: Carbonyl reductase 1; DDP: cisplatin; EMT: mesenchymal transition; GPX4: glutathione peroxidase 4; GSH: glutathione; HMOX1: heme oxygenase 1; LIP: labile iron pool; LPO: lipid peroxidation; L-ROS: lipid reactive oxygen species; MDA: malondialdehyde; TBK1: tank-binding kinase; TXNRD: thioredoxin reductase.

contributing to DDP resistance, whereas the induction of novel forms of cell death, such as ferroptosis, has been shown to effectively kill DDP-resistant cancer cells that evade apoptosis[41]. The combination of DDP with Shikonin, a hydroxy-1,4-naphthoquinone isolated from *Lithospermum erythrorhizon* Sieb. et Zucc.(Boraginaceae), promotes Fe^{2+} accumulation by upregulating heme oxygenase 1 (HMOX1), initiating ferroptosis in DDP-resistant ovarian cancer cells[42]. Piperlongumine, an alkaloid derived from long pepper (*Piper longum* L.), exhibits anticancer activity in lung cancer cells by targeting the glutathione regeneration

enzyme, thioredoxin reductase 1 (TXNRD1). Although it does not induce ferroptosis, it can significantly enhance erastin-induced LPO[43].

In addition to ferroptosis-related genes and proteins, some natural compounds induce ferroptosis via regulation of the Nrf2 signaling pathway. Withaferin A, a steroidal lactone isolated from the medicinal plant Ashwagandha [*Withania somnifera* (L.) Dunal], and Tiliroside, a flavonoid found in the herbs of *Tribulus terrestris* L., both modulate the Kelch-like ECH-associated protein 1 (Keap1)/Nrf2 pathway in hepatocellular carcinoma. Withaferin A enhances sorafenib sensitivity in sorafenib-resistant hepatocellular carcinoma cells by regulating the Keap1/Nrf2-associated epithelial-to-mesenchymal transition (EMT) and ferroptosis[44]. Additionally, tiliroside synergistically combines with sorafenib to inhibit Tank-binding kinase (TBK1) activity, prompting Keap1-mediated Nrf2 ubiquitination and degradation, leading to ferroptosis in hepatocellular carcinoma cells[45]. Ginkgetin, a natural biflavonoid isolated from the leaves of *Ginkgo biloba* L., has been found to induce ferroptosis in non-small cell lung cancer cells through decreased expression of xCT and GPX4, decreased GSH/glutathione disulfide (GSSG) ratio, and inactivation of the Nrf2/HMOX1 axis. Interestingly, it can promote DDP-induced anticancer activity, which is also a result of ferroptosis induction[46].

As a key regulator of iron homeostasis, ferritin plays a vital role in storing intracellular free iron and is involved in ferritinophagy, a form of autophagic ferroptosis. In ferritinophagy, the autophagic cargo receptor nuclear receptor coactivator 4 (NCOA4) binds to ferritin heavy chains (FTH1) and delivers it to autophagosomes for degradation and iron release[47]. Human carbonyl reductase 1 (CBR1) contributes to gemcitabine resistance in pancreatic cancer. The upregulation of CBR1 induced by gemcitabine inhibits the antitumor effects of the drug. Conversely, reducing CBR1 activity enhances the sensitivity of cancer cells to gemcitabine, thereby improving its therapeutic efficacy. Chrysin, a natural bioflavonoid compound, has been discovered to induce ferritinophagy, thus enhancing gemcitabine sensitivity in pancreatic cancer cells. In Chrysin-treated cells, there is a deregulation of FTH1 and an increase in intracellular free iron levels, followed by the inhibition of CBR1, which is involved in the induction of ferroptosis[48].

The tumor suppressor protein p53 also plays an essential role in ferroptosis in certain cancers. As a diterpenoid extracted from *Torreya nucifera*, kayadiol exhibits anticancer properties through p53-mediated ferroptosis in NK/T lymphoma cells, and it could synergistically combine with L-asparaginase and DDP[49]. Artesunate (AST), another derivative of artemisinin, was found to inhibit the growth of sunitinib-resistant renal cell carcinoma cells by both inhibiting cell cycle progression and inducing ferroptosis. Interestingly, the induction of ferroptosis was associated with its inhibitory effect only in renal cell carcinoma cells expressing p53, suggesting that AST induces p53-dependent ferroptosis[50].

NECROPTOSIS

Overview of necroptosis

Necroptosis, first described in 2005, is a form of cell death characterized by morphological features similar to necrosis, including a lack of nuclear chromatin, organelle swelling, and cell membrane disruption[51]. However, unlike necrosis, which is a passive and non-programmed form of cell death, necroptosis can be regulated by multiple signal transduction pathways[52].

The classical form of necroptotic cell death is mediated by tumor necrosis factor-α (TNF-α). Initially, TNF-α binds to its specific receptor TNF receptor 1 (TNFR1), promoting its trimerization and facilitating the recruitment of several proteins including receptor-interacting protein 1 (RIP1) kinase, TNF-α receptor-associated death domain (TRADD), cellular inhibitor of apoptosis 1 (cIAP1), TNFR-associated factor 2 (TRAF2), and TNFR-associated factor 5 (TRAF5) to form complex I. Within complex I, RIP1 can

undergo polyubiquitination by TRAF2, TRAF5, cIAP1, and cIAP2. The ubiquitination status of RIP1 determines whether complex I activates the nuclear factor-κB (NF-κB) pathway to promote cell survival, or triggers cell death[53].

When the ubiquitylation of RIP is impaired, complex I can transform into complex IIa and complex IIb, leading to cell death[54]. Complex IIa can trigger caspase-8-dependent apoptosis in the absence of RIP1, whereas complex IIb depends on RIP1 for caspase-8 activation due to its deficiency in TRADD compared to complex IIa[55]. Additionally, complex IIa can transform into complex IIb. When the levels of receptor-interacting protein 3 (RIP3) and mixed lineage kinase domain-like protein (MLKL) are sufficiently high and caspase-8 is blocked, complex IIb may develop into a necrosome. Following the phosphorylation of RIP1 and RIP3, two core components of the necrosome, the activated RIP3 can further recruit and phosphorylate MLKL, triggering its oligomerization. The oligomerized MLKL is then translocated to the plasma membrane, increasing its permeability and ultimately leading to necroptotic cell death[56] [Figure 2]. In addition to TNF superfamily receptors, various other types of receptors, such as Toll-like receptors, T-cell receptors, and interferon receptors, also contribute to the activation of necroptosis[57].

Regulation of necroptosis for anti-drug resistance in cancer

As independent of caspase activation and involving distinct components from apoptotic pathways, necroptosis is an effective mechanism to overcome apoptosis resistance in cancer. However, some necroptotic core components are always lacking in cancer cells, resulting in the evasion of this mechanism. RIP3 is silenced in numerous cancer types, and this silencing is likely ascribed to genomic methylation near the RIP3 transcriptional start site or driven by oncogenes BRAF and AXL[58]. Current studies have revealed that demethylation treatment can activate necroptotic pathways by restoring the expression of RIP3. Moreover, upregulating RIPK3 expression can enhance the sensitivity of colon cancer cells to 5-FU and lung cancer cells to DDP through mediating necroptosis[59,60].

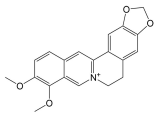
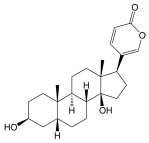
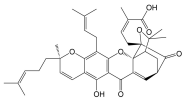
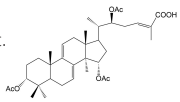
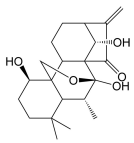
Furthermore, the low expression of MLKL appears to be associated with a poor patient prognosis in certain cancers, making it a potential novel potential prognostic biomarker for these cancers[61]. In addition, some compounds can induce MLKL-mediated necroptosis without the phosphorylation of RIP1 and RIP3, which presents a very promising prospect for future studies[62].

In addition to modulating necroptosis genes and proteins, increasing glycolytic metabolism may confer resistance to necroptosis in cancer cells under hypoxic conditions. The mechanism involves the suppression of RIP-dependent necroptosis through pyruvate scavenging of mitochondrial superoxide[63]. Several studies have suggested that inhibiting glycolysis may be a potential mechanism for necroptosis induction. For instance, selenite-induced necroptosis in prostate cancer resulted from the inhibition of glycolysis through adenosine triphosphate (ATP) depletion and phosphofructokinase activity reduction[64].

Natural compounds inducing necroptosis for cancer treatment

So far, many natural compounds can induce necroptosis in diverse cancer types, and some can also inhibit drug resistance [Table 2]. As mentioned above, modulating necroptosis core proteins is critical for necroptosis induction. By regulating the RIP1/reactive oxygen species (ROS)-mediated pathway, bufalin, an endogenous cardiotonic steroid, can induce necroptosis in adriamycin-resistant triple-negative breast cancer cell lines[65]. Numerous studies have indicated that when 5-FU is in combination with other anticancer agents, its therapeutic efficacy can be effectively enhanced[66]. Gambogenic acid is one of the main components of Gamboge which can be used in combination with 5-FU to upregulate necroptosis-related proteins such as RIP1 in lung cancer cells, thereby inducing necroptosis[67]. Piperlongumine can also activate RIP1 to produce excessive ROS, triggering necroptosis in DDP-resistant bladder cells[68]. Berberine can

Table 2. Natural compounds for anticancer drug resistance by inducing necroptosis

Compounds	Origin	Structure	Cancer	Anti-drug resistant effects	Refs
Berberine	*Coptis chinensis* Franch		Ovarian cancer	Synergizing with DDP to induce necroptosis by activating the RIP3/MLKL pathway.	[69]
Bufalin	*Bufo bufo gargarizans* Cantor		Triple-negative breast cancer	Inducing necroptosis in adriamycin-resistant triple-negative breast cancer through mediating the RIP1/ROS pathway.	[65]
Gambogenic Acid	*Garcinia hanburyi* Hook. f.		Lung cancer	Synergizing with 5-FU to induce necroptosis by increasing the expression of RIP1.	[67]
Ganoderic acid T	*Ganoderma lucidum* (Leyss.ex Fr.) Karst.		Cervical cancer	Increasing the radiosensitivity of cervical cancer by inducing necroptosis via ROS generation and increased expression of RIP and MLKL.	[71]
Oridonin	*Isodon rubescens* (Hemsley) H. Hara		Renal carcinoma	Inducing necroptosis in renal carcinoma to enhance the antitumor effects of 5-FU via ROS generation, GSH depletion, and activation of p38, ERK, and JNK.	[70]

DDP: Cisplatin; ERK: signal-regulated kinase; FU: fluorouracil; GSH: glutathione; JNK: c-Jun N-terminal kinase; MLKL: mixed lineage kinase domain-like protein; RIP: receptor-interacting protein; ROS: reactive oxygen species.

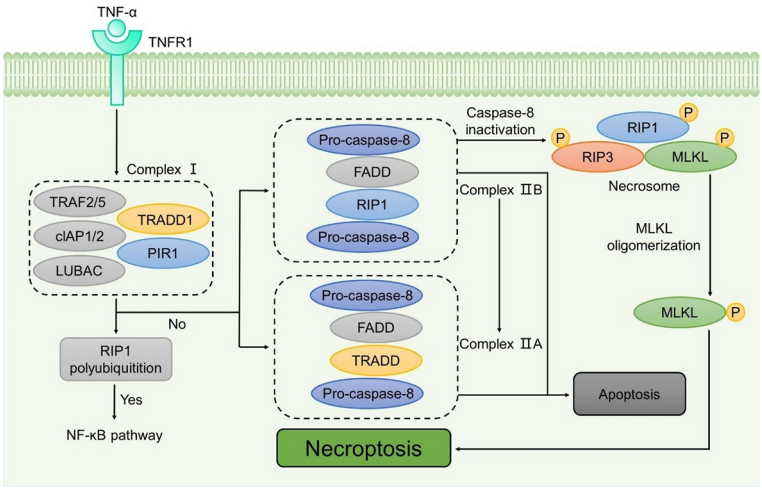


Figure 2. The classical pathway of necroptosis. The classical form of necroptosis begins with the binding of TNF-α to TNFR1, initiating the formation of complex I. If the ubiquitylation of RIP1 within complex I is inhibited, it undergoes a transition into complex IIA and complex IIB. When caspase-8 is inactive, complex IIB recruits RIP3 to form the necrosome. Once RIP3 is phosphorylated, it recruits and phosphorylates MLKL, leading to its oligomerization. The oligomerized MLKL is subsequently translocated to the plasma membrane, resulting in necroptosis. MLKL: Mixed lineage kinase domain-like protein; PIR1: receptor-interacting protein 1; RIP3: receptor-interacting protein 3; TNF: tumor necrosis factor; TNFR: TNF receptor; TRADD: TNF-α receptor-associated death domain TRAF: TNFR-associated factor.

effectively enhance the antitumor effect of DDP in ovarian cancer by increasing the expression and activation of RIP3 and MLKL, thereby inducing necroptotic cell death[69]. In TNF-α induced necroptosis, MLKL is a key downstream component of RIP1 and RIP3. However, it is worth mentioning that tanshinol A, a phenolic compound extracted from *Salvia miltiorrhiza* Bunge, can trigger non-canonical necroptosis

mediated by MLKL in lung cancer independently of RIP1 and RIP3[62].

MAPK signaling pathways also play a role in necroptosis induction. For instance, oridonin, a diterpenoid derived from *Isodon rubescens* (Hemsley) H. Hara, has been shown to enhance the cytotoxicity of 5-FU in renal cancer cells by inducing necroptosis. This process is associated with the activation of c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK)[70].

Moreover, certain studies have demonstrated that the inhibition of caspases may cause a switch from apoptosis to necroptosis in specific cancer types. Ganoderic acid T (GAT) is a triterpene of *Garcinia hanburyi* Hook. f., inducing both necroptosis and apoptosis in cervical cancer cells. Interestingly, the percentage ratio of necroptosis is increased following the increase of GAT concentration, as GAT can reduce the matrix metalloproteinase (MMP) and ATP levels and caspase-8 expression under radiation conditions[71].

Notably, glycolysis suppression has also emerged as an effective mechanism for necroptosis induction by natural compounds. Docetaxel is indeed a valuable chemotherapeutic agent utilized in the treatment of prostate cancer, primarily by inducing cell death[72]. However, similar to DDP, the effectiveness of docetaxel-induced apoptosis can also be hindered through various pathways, including the p38/p53/p21 pathway, USP33-DUSP1-JNK pathway, and PI3K/Akt/NF-κB pathway[73-75]. Therefore, several studies are currently focused on identifying alternative cell death pathways, such as necroptosis, that can be induced in docetaxel-resistant cancer cells as well. Shikonin has been extensively studied as a natural necroptosis inducer in various cancer types, and it has been demonstrated to overcome drug resistance to docetaxel in prostate cancer and DDP in bladder cancer[76,77]. Furthermore, shikonin can induce glycolysis suppression in glioma cells, which is closely associated with the accumulation of intracellular H_2O_2 triggered by the activation of RIP1 and RIP3[78].

PYROPTOSIS

Overview of pyroptosis

Pyroptosis, a pro-inflammatory programmed cell death, was originally termed by Cookson *et al.* in 2001[79]. The term derives from the Greek roots "pyro", which relates to fire or fever, and "ptosis", denoting a falling, reflecting its nature. Pyroptosis shares certain characteristics with apoptosis, such as DNA fragmentation, nuclear condensation, and caspase dependence. However, cells undergoing pyroptosis differ in that they retain intact nuclei and exhibit pore formation in the plasma membrane[80]. An increasing understanding of pyroptosis has revealed that this type of cell death can be divided into classical and non-classical pathways.

The classical pyroptotic pathway is mediated by inflammasome assembly, which consists of pattern recognition receptors (PRRs), apoptosis-related speck-like protein (ASC), and pro-caspase-1[81]. PRRs, functioning as inflammasome sensors, recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[82]. Subsequently, they recruit the bridging protein ASC, which contains a pyrin domain (PYD) and a caspase activation and recruitment domain (CARD), through specific PYD-PYD interactions. After being recruited, ASC can interact with and activate pro-caspase-1 via CARD-CARD interactions[83]. Some PRRs containing CARD can also directly bind to pro-caspase-1, forming inflammasomes without the participation of ASC[84]. Activated caspase-1 facilitates the maturation of inflammatory cytokines interleukin (IL)-1β and IL-18, as well as cleaves the pore-forming protein gasdermin D (GSDMD) to produce its N-terminal fragment (N-GSDMD)[85]. N-GSDMDs then translocate to the plasma membrane and form pores, promoting the release of mature IL-1β and IL-18. As the number of N-GSDMD pores increases, cells swell and rupture, resulting in pyroptotic cell death[86].

In non-classical pathways, caspase-3/8 can also trigger pyroptosis by activating GSDMD or GSDME[87,88]. Additionally, caspase-8 can induce GSDMC-dependent pyroptosis as well[89]. The cleavage of GSDMD can also be mediated by caspase-4/5/11, which recognizes intracellular LPS to activate the non-canonical inflammasome[90]. Notably, GSDMB can not only be cleaved by caspase-1 to directly initiate pyroptosis, but also enhance caspase-4 activity to promote this cell death mechanism[91]. Recently, the cleavage of GSDMA has been found to be catalyzed by streptococcal pyrogenic exotoxin B (SpeB), a cysteine protease secreted by group A Streptococcus. This finding demonstrates that GSDMA can also play a role in pyroptosis by releasing the cleaved N-terminal fragments, which can bind to and disrupt specific acidic lipid-containing membranes[92] [Figure 3].

Induction of pyroptosis for anti-drug resistance in cancer

In cancer treatment, the induction of pyroptosis is increasingly recognized as a promising strategy for overcoming drug resistance. NOD-like receptor family pyrin domain-containing 3 (NLRP3), a crucial inflammasome sensor in the NLR family, is recognized as a downstream target of multiple microRNAs (miRNAs) associated with cancer drug resistance. For example, by downregulating the expression of miR-556-5p in non-small cell lung cancer, NLRP3 inflammasome-mediated pyroptosis can be triggered, thereby enhancing DDP sensitivity[93].

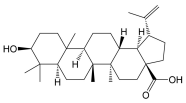
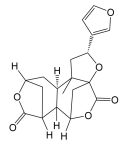
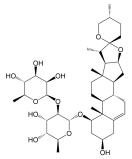
The pore-forming proteins GSDMD and GSDME, extensively studied in the GSDM family, are potential targets for combating drug resistance and contributing to the treatment and prognosis of various cancers. Some chemotherapeutic agents have been demonstrated to exert antitumor activity when used alone or in combination to induce GSDMD-dependent pyroptosis, enhancing the efficacy of chemotherapy. For instance, the co-administration of paclitaxel and ruthenium complexes can induce cell death in paclitaxel-resistant cervical cancer cells by mediating Caspase-1/GSDMD-dependent pyroptosis[94]. GSDME-mediated pyroptosis has been observed to improve the sensitivity of various drugs across different cancer types, including increasing DDP sensitivity in esophageal squamous cell carcinoma cells and oxaliplatin sensitivity in colon cancer cells[95]. Additionally, it can alleviate the side effects of DDP in patients with oral squamous cell carcinoma.

In recent years, programmed death-ligand 1 (PD-L1), known as an immune checkpoint, has emerged as a research hotspot in tumor immunotherapy. However, PD-L1 has an additional role as a non-immune checkpoint by regulating the non-classical pyroptosis pathway mediated by GSDMC/caspase-8[96]. In this way, a variety of antibiotics can induce pyroptosis in cancer cells, indicating that it could be a novel strategy to combat antibiotic resistance in chemotherapy.

Natural compounds inducing pyroptosis for cancer treatment

Nowadays, the caspase-1/GSDMD and caspase-3/GSDME pathways have attracted significant attention in pyroptosis induction, and many natural compounds have been found to activate these pathways in cancers [Table 3]. Wedelolactone, an ingredient of *Eclipta prostrata* (L.) L., can simultaneously activate these two pathways by strongly increasing the activation of caspase-1, caspase-3, GSDME and GSDMD in retinoblastoma cells[97]. Ophiopogonin B, derived from *Dioscorea bulbifera* L., can induce caspase-1/GSDMD-dependent pyroptosis in lung cancer cells, especially exhibiting a more significant suppression of growth in DDP-resistant cancer cells[98]. As a pentacyclic triterpene compound of lupine, betulinic acid can induce caspase-1-dependent pyroptosis, thereby enhancing chemosensitivity to DDP in esophageal cancer cells[99].

Table 3. Natural compounds for anticancer drug resistance by inducing pyroptosis

Compounds	Origin	Structure	Cancer	Anti-drug resistant effects	Refs
Betulinic acid	*Betula platyphylla* Suk.		Esophageal cancer	Enhancing the antitumor effects of DDP by inducing pyroptosis via increasing the levels of ASC and caspase-1 and decreasing the levels of Ki67, PCNA, SOX2, and OCT4.	[99]
Diosbulbin B	*Dioscorea bulbifera* L.		Gastric cancer	Increasing DDP-sensitivity in gastric cancer by inducing pyroptosis via regulating the PD-L1/NLRP3 pathway.	[106]
Ophiopogonin B	*Ophiopogon japonicus* (L. f.) Ker-Gawl.		Lung cancer	Increasing DDP-sensitivity in gastric cancer by inducing pyroptosis via regulating the caspase-1/GSDMD pathway.	[98]

ASC: Apoptosis-related speck-like protein; DDP: cisplatin; GSDMD: gasdermin D; NLRP3: NOD-like receptor family pyrin domain-containing 3; OCT4: octamer-binding transcription factor-4; PCNA: proliferating cell nuclear antigen; PD-L1: programmed death-ligand 1; SOX2: SRY-related high mobility group box protein-2.

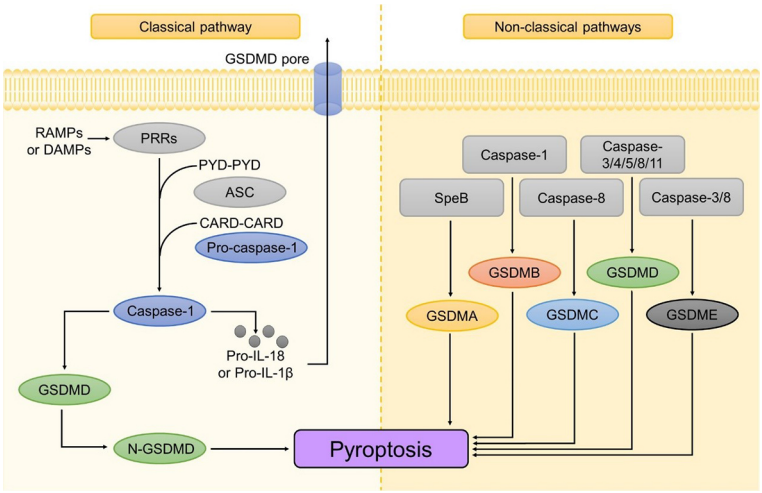


Figure 3. In the classical pathway, PRRs recognize PAMPs and DAMPs, initiating the recruitment of ASC and pro-caspase-1. The activation of caspase-1 results in cleavage of GSDMD, forming GSDMD pores that ultimately trigger pyroptosis. Furthermore, caspase-1 also results in the maturation of IL-1β and IL-18, which are eventually released from the GSDMD pores. Alternatively, in the non-classical pathway, pyroptosis can be initiated by other members of the GSDM family. CARD: Caspase activation and recruitment domain; DAMPs: damage-associated molecular patterns; GSDMD: gasdermin D; IL: interleukin; PAMPs: pathogen-associated molecular patterns; PRR: pattern recognition receptor; PYD: pyrin domain.

By activating the caspase-3-dependent pathway, natural compounds such as curcumin, dihydroartemisinin, and germacrone can induce pyroptosis in different cancers[100-102]. Some studies have reported that the activation of caspase-9 is also involved in caspase-3-mediated pyroptosis. For example, alantolactone, a terpenoid of *Inula helenium* L., can promote the cleavage of caspase-9 and caspase-3 to induce GSDME-mediated pyroptosis in anaplastic thyroid cancer[103]. Some chemotherapeutic agents, such as DDP, 5-FU, and carboplatin, have been demonstrated to combat cancer drug resistance by inducing GSDME-dependent pyroptosis, while whether natural compounds can also suppress drug resistance through this pathway needs further investigation[95,104,105].

In addition, by downregulating PD-L1 to activate NLRP3-mediated pyroptosis, Diosbulbin B extracted from *Dioscorea bulbifera* L. can sensitize DDP-resistant gastric cancer cells to DDP[106]. At present, there are few reports on whether natural compounds induce pyroptosis via the regulation of other GSDM family proteins. This is partly because of their unclear functions in initiating pyroptotic cell death. Further elucidation of how the other members of the GSDM family contribute to pyroptosis induction in cancers may provide new insights for the search for natural compounds with anticancer and anti-drug resistance activities.

PARAPTOSIS

Overview of paraptosis

Paraptosis was first introduced as a form of programmed cell death by Sperandio *et al.* in 2000[107]. It derives from "para", meaning "next to" or "related to", and "apoptosis", suggesting that it is distinct from apoptosis. The main morphological features of paraptosis include cytoplasmic vacuolization, swelling of the endoplasmic reticulum and/or mitochondria, and the absence of nuclear fragmentation or apoptotic body formation. In this paradigm, caspases are not activated, and thus, cells undergoing paraptosis are resistant to caspase inhibitors[108].

The MAPK pathways play a critical role in paraptosis, and research focusing on the ERK, JNK, and p38 pathways is particularly extensive. Notably, the protein AIP1/Alix was described as the first specific inhibitor of paraptosis, capable of restraining the insulin-like growth factor-I receptor (IGFIR)-induced paraptotic process mediated by the MAPK/ERK and JNK pathways[109]. Furthermore, TrxR1 inhibition and GSH depletion have been observed to potentially activate the MAPK pathways by triggering the accumulation of cellular ROS[110]. Paraptosis induction is also related to the homeostasis of intracellular Ca^{2+}, which is mainly regulated by the endoplasmic reticulum and mitochondria. Intracellular Ca^{2+} can be released from the endoplasmic reticulum into mitochondria when paraptosis is initiated, resulting in the endoplasmic reticulum and mitochondrial dilation and ultimately leading to cell death. The voltage- and Ca^{2+}-activated K^+ (BKCa) channels are widely expressed in body and have the ability to link changes in intracellular calcium to outward hyperpolarizing potassium currents. The activation of these channels will disrupt the osmotic balance, initiating cell swelling and vacuolization[111]. Additionally, proteasome inhibition may also promote paraptosis by inducing endoplasmic reticulum (ER) stress[112] [Figure 4].

Paraptosis potential applications in cancer drug resistance

Paraptosis induction is a potential strategy for developing non-genetically modified tumor vaccines. In rat T9 glioma cells, the activation of BKCa channels promotes the overexpression of heat shock proteins and the translocation of HMGB1 from the nuclear region to the periphery, stimulating immune responses and initiating paraptosis. Rats injected with paraptotic T9 glioma cells, which are killed by prolonged BKCa channel activation, can develop specific immunity to T9 cells. This suggests the potential of using these treated cells as a functionally killed vaccine[113].

Due to its unique molecular mechanism, paraptosis induction also contributes to enhancing the activity of proteasome inhibitors in cancer cells. Although many proteasome inhibitors have shown antitumor activities, their clinical efficacy is unsatisfactory as their effectiveness can be compromised by both primary and secondary resistance mechanisms. Therefore, combination therapy can be seen as an effective strategy to address proteasome inhibitor resistance. For instance, by triggering paraptotic cell death, bortezomib (Btz), a 20S core particle inhibitor of the proteasome, can be combined with loperamide, an antidiarrheal agent, to enhance Btz sensitivity and reduce its side effects, effectively combating the colon cancer[114].

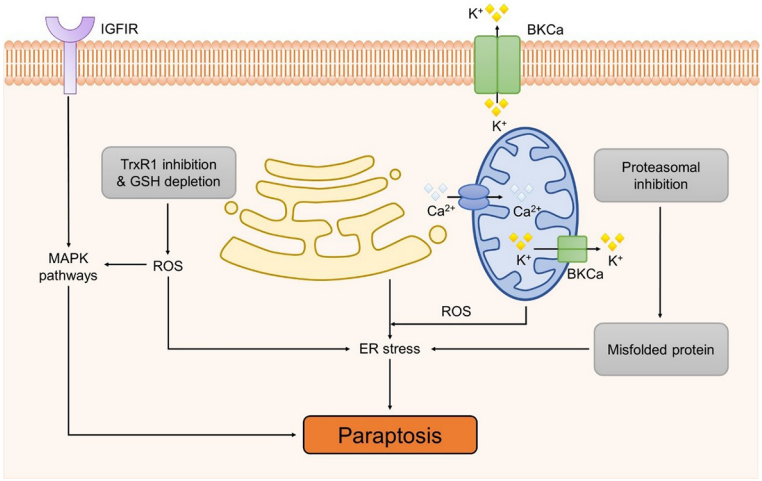


Figure 4. The mechanism of Paraptosis. Paraptosis was initially discovered to be induced by IGFIR and mediated through the MAPK pathways. Furthermore, it is intricately linked to various factors, including TrxR1 inhibition, GSH depletion, intracellular Ca^{2+} homeostasis, activation of BKCa channels, and proteasome inhibition. BKCa: Ca^{2+}-activated K^+; ER: endoplasmic reticulum; GSH: glutathione; IGFIR: insulin-like growth factor-I receptor; MAPK: MAP kinase; ROS: reactive oxygen species.

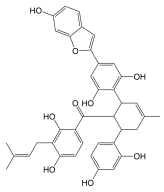
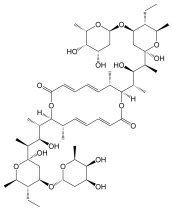
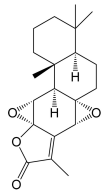
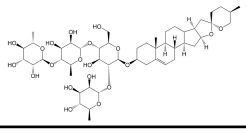
Natural compounds inducing paraptosis for cancer treatment

Currently, a number of natural compounds have shown potential in cancer treatment by inducing paraptosis [Table 4], with many of them modulating the MAPK signaling pathways to trigger ER stress. One such example is Paris Saponin II, derived from *Paris polyphylla* Smith, which effectively induces paraptosis by activating the JNK pathway and augmenting ER stress[115]. Moreover, the activation of this paraptosis-associated pathway enhances the sensitivity of DDP in lung cancer. Jolkinolide B, an active abietane ent-diterpenoid, is also a noteworthy compound that induces paraptosis in both sensitive and DDP-resistant bladder cancer cells by activating the ERK pathway and enhancing ER stress[110]. Additionally, Chalcomoracin, isolated from *Morus alba* L., has been discovered to enhance the sensitivity of non-small cell lung cancer to radiation by augmenting ER stress[116]. Elaiophylin is a natural antibiotic derived from *Streptomyces melanosporus* that can also induce paraptosis through the hyperactivation of the MAPK pathway. This compound demonstrates notable efficacy in eliminating ovarian cancer cells that are resistant to multiple drugs, including platinum, taxane, and poly (ADP-ribose) polymerase inhibitor (PARPi)[117].

Some natural compounds such as curcumin, morusin, and ophiobolin A can also induce paraptosis by affecting ion homeostasis. Curcumin can induce paraptosis in epithelial ovarian cancer mainly through mitochondrial Ca^{2+} overload, which subsequently contributes to mitochondrial swelling and dysfunction[118]. Similarly, morusin, a prenylflavonoid, can also induce paraptosis in breast cancer by triggering mitochondrial Ca^{2+} overload[119]. In glioblastoma cells, paraptosis induced by ophiobolin A, a sesterterpenoid phytotoxin from the genus Bipolaris, is linked to K^+ homeostasis imbalance, primarily caused by the blockage of BKCa channels[120].

Furthermore, natural compounds can induce paraptosis by disrupting sulfhydryl homeostasis and suppressing proteasome functions, both of which can be significantly inhibited by thiol antioxidants. In this way, plumbagin, extracted from *Plumbago zeylanica* L., can induce paraptotic cell death in different cancer types[121]. Paraptosis can also be triggered in a p53-dependent manner. For instance, Ginsenoside Rh2, which is a bioactive product in *Panax ginseng* C. A. Meyer, can induce paraptosis in colorectal cancer via activating the p53 pathway as well as the NF-κB survival pathway[122].

Table 4. Natural compounds for anticancer drug resistance by inducing paraptosis

Compounds	Origin	Structure	Cancer	Anti-drug resistant effects	Refs
Chalcomoracin	*Morus alba* L.		Non-small cell lung cancer	Increasing the radiosensitivity of non-small cell lung cancer by inducing ER stress-mediated paraptosis via activation of the MAPK pathway.	[116]
Elaiophylin	*Streptomyces hygroscopicus*		Ovarian cancer	Inducing paraptosis to overcome platinum, taxane, and PARPi resistance in ovarian cancer by regulating the SHP2/SOS1/MAPK pathway.	[117]
Jolkinolide B	*Euphorbia fischeriana* Steud		Bladder cancer	Inducing ROS-mediated paraptosis to suppress the growth of DDP-resistant bladder cancer by targeting thioredoxin and glutathione systems.	[110, 131]
Paris saponin II	*Paris polyphylla* Smith		Non-small cell lung cancer	Enhancing the antitumor effects of DDP by regulating the JNK pathway.	[115]

DDP: Cisplatin; ER: endoplasmic reticulum; JNK: c-Jun N-terminal kinase; MAPK: MAP kinase; PARPi: poly (ADP-ribose) polymerase inhibitor; ROS: reactive oxygen species; SHP2: src homology 2 domain-containing tyrosine phosphatase 2; SOS1: son of sevenless homolog 1.

CONCLUSION

Numerous natural compounds possess the ability to elicit anticancer and anti-chemoresistance effects by triggering non-apoptotic cell death mechanisms such as ferroptosis, necroptosis, pyroptosis, and paraptosis. It is noteworthy that while these forms of cell death have unique regulators, some common pathways can also govern them. This implies that multiple cell death pathways may occur concurrently and be subject to simultaneous regulation. In 2019, Malireddi *et al.* introduced PANoptosis, an innovative form of cell death that amalgamates essential features of pyroptosis, apoptosis, and necroptosis yet defies a straightforward classification under any one of these categories[123]. This discovery underscores the co-regulation and interplay among these cell death pathways, suggesting that drugs may possess multifaceted regulatory effects by modulating master factors within these pathways. Beyond the four aforementioned types of cell death, some other novel cell death mechanisms have surfaced in recent years, including parthanatos, disulfidptosis, and cuproptosis[124-126]. These revelations open up new avenues for identifying targets in cancer treatment and provide additional strategies for combating cancer drug resistance. As such, ongoing exploration is essential to ascertain whether natural compounds can elicit anticancer effects through these emerging forms of cell death.

DECLARATIONS

Authors' contributions

Conceptualization: Chen GQ, Chen S, Chen JW

Data curation and original draft preparation: Chen JW, Chen GQ, Chen S

Figures and Tables: Chen JW, Chen GQ, Chen S
Review and editing: Chen GQ, Chen S, Chen JW
All authors contributed to the article and approved the submitted version.

Availability of data and materials
Not applicable.

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Conflicts of interest
All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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Cancer Drug Resistance

Review

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Unlocking antitumor immunity with adenosine receptor blockers

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Abstract

Tumors survive by creating a tumor microenvironment (TME) that suppresses antitumor immunity. The TME suppresses the immune system by limiting antigen presentation, inhibiting lymphocyte and natural killer (NK) cell activation, and facilitating T cell exhaustion. Checkpoint inhibitors like anti-PD-1 and anti-CTLA4 are immunostimulatory antibodies, and their blockade extends the survival of some but not all cancer patients. Extracellular adenosine triphosphate (ATP) is abundant in inflamed tumors, and its metabolite, adenosine (ADO), is a driver of immunosuppression mediated by adenosine A2A receptors (A2AR) and adenosine A2B receptors (A2BR) found on tumor-associated lymphoid and myeloid cells. This review will focus on adenosine as a key checkpoint inhibitor-like immunosuppressive player in the TME and how reducing adenosine production or blocking A2AR and A2BR enhances antitumor immunity.

Keywords: Immunotherapy, adenosine, adenosine receptors, adenosine A2A receptors (A2AR), adenosine A2B receptors (A2BR), tumor cells, immune cells, tumor microenvironment

INTRODUCTION

Deadly tumors have the ability to resist the body's formidable immune defenses. They create protective micro-environments that limit antigen presentation, inhibit T and natural killer (NK) cell responses, and

induce T cell exhaustion, effectively escaping immune surveillance. These mechanisms allow some tumors to grow unchecked and resist conventional cancer therapies.

Checkpoint inhibitors that block immunosuppressive signaling molecules such as PD-1, T lymphocyte antigen 4 (CTLA4), and lymphocytic activation gene 3 protein (LAG-3) have ushered in a new era of cancer immunotherapy, offering hope for prolonged survival and enhanced quality of life for many patients. However, the beneficial effects of these therapies are not universal due to the ability of some tumors to maintain an immunosuppressive environment. The interplay between cancer cells and immune cells within the tumor microenvironment (TME) is a critical determinant of the therapeutic response.

A key driver of immunosuppression within the TME is extracellular adenosine (ADO), an adenosine triphosphate (ATP) metabolite. ADO formation and its immunosuppressive signaling play a pivotal role in maintaining the immunosuppressive state of the TME, promoting tumor growth, and facilitating resistance to other therapies. This review explores the role of ADO signaling in the TME. Inhibiting ADO receptors on immune cells reduces immunosuppression and, in some cases, has an additive antitumor effect when combined with other cancer treatments.

UNDERSTANDING NON-RESPONDERS TO IMMUNOTHERAPY IN SOLID TUMORS

Despite having antitumor effects, cancer immunotherapy often fails. One prominent reason is that most tumor proteins are recognized as self-proteins and fail to activate T cells, which serve as the frontline warriors of the adaptive immune response[1]. To the extent that tumors are recognized by the immune system, their activation is muted by immunosuppressive signals like adenosine.

Antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) also regulate the immune response to tumors[2]. The function of APC is to recognize, engulf, and present tumor antigens on their surface. Their failure to optimally phagocytose and present antigens can undermine the initiation of adaptive immunity. The host's baseline immune response also significantly influences the success or failure of cancer immunotherapy. The baseline immune response is crucial in determining treatment efficacy, represented by the association between increased T cell infiltration into tumors and improved patient survival and immunotherapy response rates[3]. However, the factors that dictate the extent of T cell infiltration into tumors, whereby an extensively infiltrated tumor is considered "hot", and a sparsely infiltrated tumor is considered "cold", are just beginning to be elucidated. The factors that influence infiltration vary across tumor types and subtypes due to immune cell heterogeneity[4]. The complexity and dynamics of the immune system, in conjunction with the adaptability of tumor cells, create a challenging landscape for the successful deployment of cancer immunotherapy.

The complexity of the TME in solid tumors

The TME in solid tumors is complex, consisting of various immune cells, cytokines, chemokines, and metabolites. Specific features of the TME depend on the tumor type and the location within the patient. Some tumors develop an extracellular matrix (ECM) of fibrous proteins and stromal cells that define and isolate the TME from the surrounding tissue[5,6]. Within "cold" solid tumors, very few antitumor CD8+ T cells, NK cells, and DCs are present, due to failure by immune cells to enter through the ECM[7]. Immune cells that contribute to the immunosuppressive state are tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). Monocytes that enter tumors can polarize into M1 (proinflammatory) or M2 (immuno-suppressive) cells. In cancer, most become M2 and MDSC[8] and function to secrete vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β), which stimulates angiogenesis within the tumor[9]. M2-TAMs and MDSCs also suppress CD8+ T cell

infiltration and increase regulatory T cell (Treg) differentiation from precursor CD4+ T cells[10]. Tregs release suppressive cytokines such as interleukin-10 (IL-10) and TGF-β that inhibit CD8+ T cell function and enhance cancer cell escape from the immune attack[11]. In some solid tumors, such as pancreatic ductal adenocarcinoma (PDAC), increased IL-10 is correlated with reduced survival[12-14].

MDSCs are considered immature myeloid cells and secrete reactive oxygen species (ROS), IL-10, IL-13, TGF-β, arginase-I, inducible nitric oxide synthase (iNOS), and other immunosuppressive factors[15-17].

The TME is usually hypoxic due to a poorly developed tumor vasculature. Most cytotoxic immune cells (CD8+ T cells and NK cells) function poorly in hypoxic states, while suppressive cells (M2 TAMs and Tregs) thrive[18]. The hypoxic TME favors the production of ADO and the induction of immunosuppressive A2A receptors (A2AR) and A2B receptors (A2BR) in immune cells. A shift in tumor metabolism from oxidative phosphorylation to primarily glycolysis also suppresses immune cell infiltration due to increased lactate in the TME. The increase in lactate lowers tumor pH[19]. This decrease in pH drives M2 polarization while inhibiting the nuclear factor of activated T cells (NFAT) within T cells[20,21]. This suppression of NFAT inhibits chemotaxis into tumors and reduces T cell activation.

The role of immune checkpoint inhibitors (ICIs) in cancer immunotherapy

Immune checkpoint inhibitors (ICIs) have greatly advanced immunotherapy, especially in some hard-to-treat tumors. The two most studied checkpoints are CTLA4 and PD-1[1,22,23]. Although antibodies that block these inhibitory signals have improved survival, most patients with solid tumors eventually develop either primary or secondary resistance to ICI. In primary resistance, tumors display early resistance to ICI and progress soon (within six months) after ICI treatment. In secondary resistance, patients respond to treatment initially but develop resistance later[1]. Studies have demonstrated that the tumor mutational burden (TMB) influences the response to ICI[24-26]. Reduced TMB within tumors treated with ICI can result in acquired resistance to ICI immunotherapy.

Contributions to therapy resistance by suppressive state and hypoxia

Suppressive immune cells within the TME contribute to checkpoint inhibitor resistance. Tregs, MDSCs, and M2-TAMs secrete immunosuppressive cytokines (TGF-β, CXCL8, CCL5, and IL-10) that prevent cytotoxic infiltrating immune cells from entering the tumor[27-31]. An increase in VEGF due to the activation of the mitogen-activated protein kinase (MAPK) pathway can also stimulate tumor angiogenesis[27-31] and inhibit immune cell infiltration[32].

Hypoxia results from various physiological and pathological conditions, including solid tumors, ischemia-reperfusion injury, stroke, and chronic obstructive pulmonary disease (COPD)[33]. A hypoxic TME contributes to an increase in extracellular ADO. The molecular mechanisms underlying hypoxia-driven responses include *Adora2a* and *Adora2b* via HIF-1α and HIF-2α[34,35]. HIFs also stimulate angiogenesis, vasodilation, and attenuation of inflammation[34,36]. HIF-1α induces CD73 and CD39 and increases the conversion of ATP into ADO, leading to T cell inhibition, metastasis, and increased angiogenesis[37-40]. The accumulation of ADO within tumor suppresses cytotoxic immune cells and APCs, such as CD8+ T cells and DCs, while enhancing the accumulation of immunosuppressive cells[41,42]. When ADO encounters its receptors, it can affect the activity of neutrophils and macrophages, reducing the release of IL-12, tumor necrosis factor-alpha (TNFα), and ROS[43-45].

The role of cancer-associated fibroblasts (CAFs) in tumor development

Cancer-associated fibroblasts (CAFs) have a different morphology than other cells within the tumor. They lack epithelial, endothelial, and leukocyte markers, and do not have the same mutations as tumor cells[46].

CAF development within tumors occurs when there is an increase in inflammatory markers such as IL-6 and TGF-β due to cancer cell DNA damage[47,48]. When IL-6 and TGF-β are increased in the TME, they tend to reduce the number of T cells, limiting the extent of the antitumor response[49]. These cytokines increase JAK-STAT signaling and ECM transition, promoting CAF formation[50-52]. Breast cancers increase Notch signaling within the TME to increase CAFs[53]. Patients who receive chemotherapy and radiation to treat solid tumors experience DNA breaks, and this stress can promote fibrosis or CAF accumulation and function. This change in CAF function causes resistance to therapy in various solid tumors[54-56].

Solid tumor CAFs may exhibit different phenotypes depending on the TME. The different phenotypes exhibit different cell surface markers, but identifying these can be challenging[46]. Breast cancers increase expression of fibroblast activation protein (FAP) to cause high immunosuppression through Treg activation[57]. Pancreatic cancers express both myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs) at different locations in the tumor. MyCAFs have high expression of TGF-β and αSMA and are located close to tumor cells, while iCAFs have high IL-6 secretion and are more distal in the TME[58,59]. iCAFs can recruit TAMs and MDSCs to the TME to increase the immunosuppressive state[13,60]. Targeting the MAPK/STAT pathways in iCAFs through inhibitors in combination with checkpoint inhibitor therapy (e.g., anti-PD-1) can lead to increased survival of patients with solid tumors like PDAC[60]. McAndrews *et al.* discovered that in early-stage PDAC, iCAFs tend to be more abundant, while myCAFs have a higher abundance in late-stage cancer. When FAP+ CAFs were depleted, there was an increase in mouse survival. Conversely, when αSMA+ CAFs were depleted, there was a decrease in survival. In the TME, when FAP+ CAFs were depleted, there was a decrease in macrophages and B cells. However, αSMA+ CAF loss showed a decrease in effector T cells (Teff) and increased Tregs. A loss in IL-6 production in FAP+ CAFs increased responses in mice to gemcitabine therapy and combination therapy of gemcitabine + checkpoint inhibitors[57].

THE ADENOSINE PATHWAY: A NEW APPROACH TO OVERCOMING THERAPEUTIC RESISTANCE TO CHECKPOINT INHIBITORS

Understanding the interaction between cells in the TME is crucial to overcoming therapeutic resistance. The concept of targeting ADO biosynthesis or inhibiting its receptors has garnered increased interest from the scientific community[58]. Targeting extracellular ADO and its receptors opens opportunities for increasing the antitumor immune response in innate and adaptive immune cell populations normally suppressed within the TME[59]. Since high ADO levels in the TME are predictive of immunosuppressive responses[60], combining current immunotherapies with ADO blockade may help to overcome ICI resistance in solid tumors.

Chemotherapies and various cancer treatments result in elevated cell death and heightened ATP release[61]. ATP is rapidly converted to ADO within solid tumors. This process is mediated by ectonucleotidases CD39 and CD73 and contributes to the formation of an immunosuppressive TME[62]. CD39 acts on ATP to produce adenosine monophosphate (AMP), which is subsequently converted into ADO by CD73. The resulting extracellular ADO interacts with one of four G-protein-coupled receptors (A1R, A2AR, A2BR, and A3R) found in tumor cells, immune cells, and endothelial cells. This increase in ADO levels within the TME hinders the activity of effector immune cells and promotes the expansion of immunosuppressive regulatory T cells[63]. Exosomes released into the TME during cell death also express CD73 and CD39[62].

The A2AR is associated with elevated levels of checkpoint molecules like PD-1, CTLA4, and LAG-3 on T cells[64]. Activation of this receptor tends to inhibit the antitumor functions of macrophages and the proliferation and cytokine production of cytotoxic T cells[65]. However, increased expression of CD39 and

CD73 within the TME leads to an upsurge in MDSCs and Tregs[59].

The influence of adenosine receptor expression in key immune cells

Innate immunity: the role of adenosine receptors in macrophages, dendritic cells, and natural killer cells

Innate immunity plays a critical role in the body's defense against cancer by providing the first line of protection against malignant cells [Figure 1]. This system, comprising various immune cells such as NKs, TAMs, DCs, and soluble factors like cytokines and chemokines, acts to identify and eliminate transformed cells. Recent studies have highlighted the dynamic interplay between innate immunity and cancer progression, shedding light on the delicate balance between tumor-promoting and tumor-suppressing functions of the innate immune system. An essential aspect of the innate immune system's interaction with cancer cells involves the A2BR, which modulates immune cell functions and significantly impacts the balance between tumor-promoting and tumor-suppressing activities.

Tumor-associated macrophages

TAMs exhibit substantial plasticity that can play a part in tumor progression and drug resistance[66]. The two main classes of TAMs within the TME are activated M1 and alternatively activated M2[67]. M1 is known to be the proinflammatory subset within the tumor, while M2 is considered to be suppressive. However, the classes are not static; the cells can change their state based on the cytokines present. There are also subsets of M2 within the TME, and each class plays a role in tumor formation and progression[67,68].

The TME regulates M1 and M2 macrophages to regulate the immune response to tumors. TAM precursors are derived from embryogenic or bone marrow-derived monocytes[66]. TAMs tend to differentiate primarily into M2-like phenotypes. These cells express high levels of VEGF (pro-angiogenic), mannose receptor (CD206), and scavenger receptor (CD163). They release suppressive cytokines such as IL-10 and promote immunosuppression within the tumor. M1 plays a key role in vaso-proliferation through the secretion of inflammatory cytokines such as IL-6, IL-8, TNF-α, and IL-1β[67,69,70]. The presence of TAMs with high levels of IL-1β within the TME contributes to neovascularization and is a predictor[69].

Apoptotic cells phagocytosed by TAMs (aka efferocytosis) release ATPs into the extracellular space in tissues[71]. ATP derivatives, specifically ADO, affect the immune activation of TAM through ADO receptors[71-73]. The A2BR is upregulated on TAMs in response to interferon-gamma (IFN-γ). When activated, A2BR suppresses the production of TNFα in infiltrating TAMs, inhibiting their capacity to secrete cytokines that are crucial for antitumor immunity. This process ultimately promotes tumor growth[71].

Dendritic cells

DCs are important APCs that present antigens to T cells on MHC proteins. T cells that recognize self-proteins abundant in tumors do not survive selection in the thymus. Intra-tumoral injections of DCs that initiate CD8+ T cell activation have been used to increase responses to immune checkpoint blockade immunotherapies[74-76]. Damage-associated molecular patterns (DAMPs) are used to determine if an immune response needs to be stimulated or if there is immune tolerance to that antigen[74,77]. DAMPs initiate a CD8+ T cell response within tumors but can also help re-prime effector CD8+ T cells to continue the adaptive immune response. However, when tumor cells overcome immune surveillance, DCs may have altered antigen processing and defective T cell activation[78].

Within the TME, there are numerous subsets of DCs along with migratory/tissue-resident DCs[79]. Classical DCs are derived from common myeloid progenitors that differentiate into common DC progenitors. Plasmacytoid DCs (pDC) are believed to be derived from lymphoid cells but can also be derived from

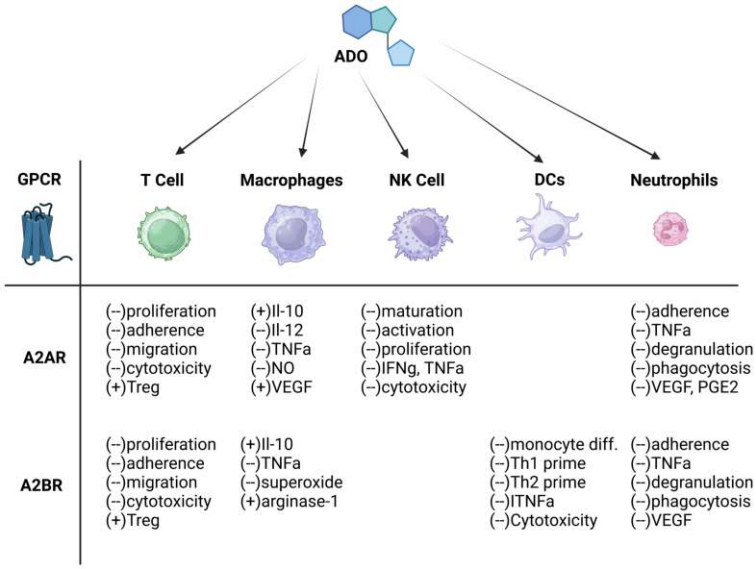

Figure 1. Adenosine's pleiotropic effects on immune cells. ADO facilitates the evasion of tumor cells from immune detection by restricting the activity of T cells, DCs, NK cells, macrophages, and neutrophils. Concurrently, adenosine amplifies the functionality of immunosuppressive cell types like MDSCs and Tregs. ADO: Adenosine; A2AR: A2A receptors; A2BR: A2B receptors; DCs: dendritic cells; MDSCs: myeloid-derived suppressor cells; NK: natural killer; TNFα: tumor necrosis factor-alpha; Tregs: regulatory T cells; VEGF: vascular endothelial growth factor.

myeloid precursors. Common monocyte precursors differentiate into a third major subtype of DCs[80]. Classical DCs have two major states: type 1 and type 2 (cDC1 and cDC2). cDC1 acts to recognize apoptotic and necrotic cell debris presented on its MHC-I receptors to activate CD8+ effector T cells. Their function helps to drive an antitumor response within the TME[75]. cDC2 are more heterogenous than cDC1 cells in tumors but are believed to play a role in recognizing exogenous tumor antigens and presenting them to CD4+ T cells on MHC-II[81]. Within cDC2 populations, there are two further subtypes: anti-inflammatory (cDC2A) and proinflammatory (cDC2B). Classical DCs are now emerging as a potential target for PD-1/PD-L1 immune checkpoint blockade. It has been demonstrated that the proper functioning of checkpoint blockade requires cis interactions with CD80 and PD-L1, as well as PD-1 and PD-L1, between T cells and the DCs[82,83].

DCs that have differentiated during exposure to ADO display diminished activity. Moreover, these DCs express high levels of angiogenic, immunosuppressive, proinflammatory, and tolerogenic factors, such as cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), interleukin-6 (IL-6), interleukin-8 (IL-8), IL-10, TGF-β, and VEGF[84,85]. These DCs depend on the upregulation of A2BR when producing these factors to promote ongoing tumor growth and increased angiogenesis for metastasis[85]. As a result, blocking A2BR can preserve the activity of DCs to present neoantigens to T cells, thereby facilitating the process of tumor cell destruction.

NK cells

NKs are derived from CD34+ hematopoietic stem cell progenitors in bone marrow. These cells kill targets that express either no or extremely low MHC-I on their surface[86,87]. NK cells have a specific killer immunoglobulin-like receptor (KIR) on their surface that recognizes MHC-I molecules. When the KIRs recognize MHC-I in self-cells, NK cells are downregulated to prevent an immune response[86,88,89]. Tumor cells tend to have low MHC-I, which can stimulate suppressed NK cells to become activated[90]. NK cells often recognize specific cancer ligands upregulated within tumors, such as MHC-I polypeptide-related

sequence A (MICA), MICB, UL-16 binding proteins, complement factor P, and platelet-derived growth factor DD[91-93].

NK cells may have an important role in tumor immunosurveillance. In some cancers, such as colon cancer and gastrointestinal stromal tumors, low NK cell expression is associated with poor outcomes[94,95]. NK cells can also kill circulating tumor cells that are implicated in metastasis[96]. Tumors may become resistant to NK cell effects by suppressing immune cell activation. Like other immune cells, the hypoxic and low-nutrient environment in tumors can decrease NK cell activation[97]. Tumors increase the interactions of activating ligands with their receptors on NK cells to stimulate later resistance to the cells. This increased stimulation can suppress the NK cell function[98].

When NK cells are activated and encounter adenosine through the A2BR, it triggers the cyclic adenosine monophosphate (cAMP) pathway. This activation subsequently blocks cytotoxic activity and cytokine production, diminishing antitumor activity[99,100]. NK cells can also increase CD73 on their surface after encountering mesenchymal stromal cells, thereby contributing to an increase in ADO and tumor growth[101]. This increase in ADO associated with high levels of A2AR contributes to suppressing NK antitumor function[102,103].

Adaptative immunity - the influence of adenosine receptors on t cell function

Adaptive immunity is the second line of defense in the immune system during infection or cancer. These immune responses are cytotoxic to tumor cells. The main two tumor-infiltrating lymphocyte (TIL) cell populations that mediate adaptive immunity are T cells (CD4+ and CD8+) and B cells. T cell infiltration within tumors depends on the tumor chemokine profile and how easily immune cells can enter through the tumor extracellular matrix. Over the last decade, improved methods have been developed to engineer T cells to be better at avoiding cancer immunosuppression. These techniques have resulted in clinical trials of blood-derived tumors and some sarcomas. Very little progress has been made for solid tumors. However, targeting the A2AR on T cells may help overcome the difficulties of T cell immunotherapies in solid tumors. Since ADO causes an increase in Tregs among infiltrating T cells, blocking A2AR can help maintain a high amount of CD4+ T cells in solid tumors. Relative to T cells, B cells have very low levels of A2BR, and limited studies have investigated its role in A2BR activation from ADO[104]. A2AR are more abundant in human than mouse B cells, but their role in immunotherapy is unknown.

T cells

T cells have been the main target of immunotherapy. CAR T cells and immune checkpoint inhibitors have been used to enhance T cell-mediated tumor killing. Dangaj *et al.* demonstrated that CCL5 must be present within the TME for TILs to enter solid tumors. The macrophages and DCs within the tumor also need to produce CXCL9 to aid in T cell infiltration[105]. Anti-PD-1 therapies have only shown limited success in solid tumors. In metastatic head and neck squamous cell carcinoma, only 15% of patients responded to anti-PD-1 treatment, and very few responses have been seen in microsatellite-stable colorectal cancer[106,107]. Duhen *et al.* discovered a subset of CD4+ T cells in tumors that are double positive for PD-1 and inducible costimulator (ICOS)[106]. These cells can have a tumor tissue-resident phenotype that allows them to recognize both tumor antigens and neoantigens on MHC-II. CD8+ TILs, on the other hand, are more heterogeneous in their response to tumor antigens[108]. The presence of PD-1 and ICOS on CD4+ T cells may work in conjunction with CD8+ T cells to stimulate a robust antitumor response.

Co-expression of CD39 and CD103 on CD8+ TIL within solid tumors has shown promise in targeting tumor cells. CD8+ T cells that have high expression of CD39 and CD103 can be identified in both primary

tumors and metastatic sites but not in the periphery[109]. The level of CD39 and CD103 double positive (DP) cells determines how well patients will respond to immunotherapy[110,111]. However, tumors can still escape these DP TILs through exhaustion mechanisms. All DP TILs express high levels of PD-1 and other exhaustion markers[112]. Checkpoint inhibitors may be useful, but there are also additional ADO pathways linked to CD39+ cells. Blocking the ADO pathway and using immune checkpoint inhibitors may help keep the DP CD8+ T cells active and prevent tumor growth.

Tumor cells increase their expression of CD39 to suppress both CD4+ and CD8+ T cell proliferation and cytotoxicity in the TME[100]. Activation of A2AR in T cells causes increased CD4+ differentiation into Treg cells. There is also an increase in additional suppressive receptors such as PD-1, LAG-3, CTLA4, and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on the T cells. Increases in ADO may have a negative effect on immunotherapies when checkpoint inhibitors are given to patients alone[113].

The importance of ADO biosynthesis in the TME

Extracellular ADO is found at low levels in unstressed tissues[114]. It is produced in response to the breakdown of adenine nucleotides and AMP outside injured cells [Figure 2][115]. In response to cancer initiation, ADO levels rapidly increase within tissues due to hypoxic, inflammatory, and/or ischemic conditions. Stressed cells release ATP into the extracellular space as a distress signal that transiently signals via P2 purinergic receptors. Ectoenzymes CD39 and CD73 can rapidly break down extracellular ATP on cell surfaces to produce extracellular ADO[116,117]. Initially, CD39 converts ATP into adenosine diphosphate (ADP) and AMP, followed by CD73-mediated conversion of AMP into ADO[118]. The accumulation of ADO in the TME helps create the suppressive niche.

Inhibition of critical immune mechanisms stimulates the formation of the pro-adenosine niche and fibrotic remodeling

The formation of solid tumors in tissues begins with an increase in cell death, inflammation, and hypoxia. This leads to an increase in extracellular ATP and ADO within the TME. When the proinflammatory metabolite extracellular ATP is cleaved into extracellular ADO and is recognized by the A2AR and A2BR within tumors, there is suppression of immune functions[59]. Endothelial cells within the forming tumor and infiltrating immune cells express CD39 and CD73 on their surface. This allows for an increase in ADO within the TME. Endothelial and immune cells also express A2BR on their surface, and when activated by ADO, the tumor can suppress immune cell infiltration. Solid tumors become hypoxic, which feeds back to increase ATP, CD73, and CD39 in the TME to further suppress the immune infiltration[115]. Tumor cells interact with suppressive immune cells to increase A2BR expression, leading to metastasis, proliferation, and VEGF production[119].

CAF increases within solid tumors, forming a dense tumor stroma. These CAFs express high levels of CD39 and CD73 on their surface in various solid tumors such as ovarian, pancreatic, colorectal, and breast cancer, which contribute to ADO production[120-122]. A dense fibrotic stroma allows ADO to remain in high concentration to drive immunosuppressive signaling throughout the tumor. An increase in A2BR on CAFs increases the secretion of IL-6 into the TME, which can convert epithelial cells to a more mesenchymal phenotype[63]. This remodeling of the TME leads to increased metastasis and therapy resistance.

Increases in the ADO pathway cause resistance to immune checkpoint inhibitor therapies

Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA4 have shown great promise for improving the survival of patients with solid tumors. This form of therapy targets PD-1 and CTLA4 on CD8+ T cells. Tumor cells inhibit CD8+ T cell function by targeting these checkpoint molecules. By blocking PD-1 and

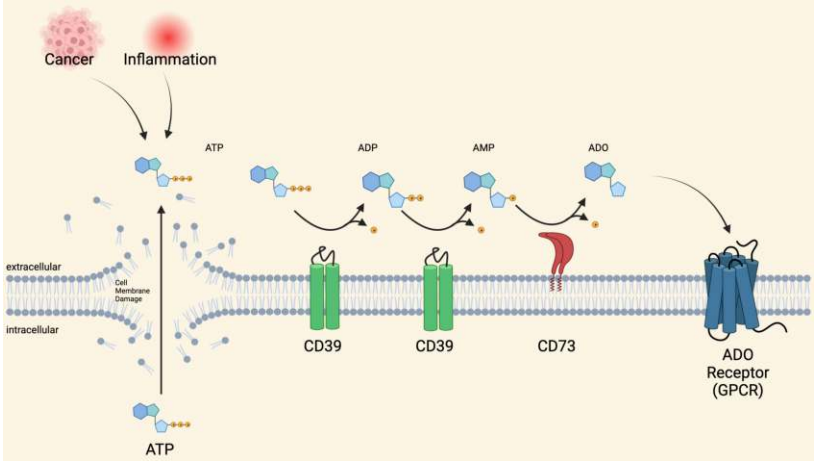

Figure 2. Adenosine Biosynthesis in Inflamed Tissues. The accumulation of extracellular ATP, driven by stress-induced conditions, stimulates extracellular ADO production by the enzymes CD39 and CD73. ADO binds four G protein-coupled receptors, A1, A2A, A2B, and A3. ADO: Adenosine; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate.

CTLA4 from being recognized, the cytotoxic function of the CD8+ T cells is increased to clear the tumor cells[113]. However, patients with solid tumors tend to relapse and become resistant to checkpoint therapy. Maj *et al.* discovered that when checkpoint therapy is given, there is an increase in the death of cancer cells and Tregs. The sudden death of these cells releases a high amount of ATP in the TME, which is then converted to adenosine by CD39 and CD73. This increase in ADO in the TME counteracts checkpoint therapy and suppresses the antitumor immune response[123].

While an increase in CD73 and CD39 is associated with poor prognosis in patients, increasing A2AR and A2BR expression also contributes to an increased risk of resistance to checkpoint therapy. It has been found that having an increase in A2AR in non-small cell lung cancer (NSCLC) or A2BR in triple-negative breast cancer (TNBC) contributes to poor survival[119,124]. Chalmin *et al.* showed that the adenosine pathway is involved in resistance to anti-PD-1 therapies. They demonstrated that when patients were given checkpoint therapy, there was an increase in CD73, leading to resistance[125]. Combination therapies targeting CD73, and checkpoint inhibitors may help overcome early resistance in solid tumors. Studies have shown that targeting CD73 and PD-1 in murine colon tumors can inhibit tumor growth[126]. The idea that targeting both the adenosine pathway and checkpoint inhibitors may overcome resistance in solid tumors gives promise to advancing immunotherapy.

Endothelial cells increase CD39 and CD73 levels during hypoxia in tumors, leading to angiogenesis

Hypoxia occurs in solid tumors and suppresses immune cell infiltration by activating hypoxia-inducible factor 1/2 (HIF1/2), IL-6, TGFβ, and TNF. Under these conditions, endothelial, tumor, and various suppressive immune cells increase CD73 and CD39 to increase ATP conversion to AMP and ADO[39,127]. Tumor cells that increase CD73 expression can generate ADO to interact with A2ARs on the tumor cells to stimulate an increase in VEGF secretion[128]. VEGF works to increase angiogenesis within the tumor and provides nutrients and oxygen for growth and metastasis [Figure 3].

Endothelial cells express CD39 within tumors to degrade ATP and promote increased neovascularization and tumor growth[129]. To have the ADO concentration needed to sustain the tumor-protective endothelial barrier, CD73 and A2BR are needed within the TME[130]. Feng *et al.* and Sun *et al.* demonstrated that inhibiting CD39 on solid tumor endothelial cells decreased angiogenesis and tumor growth[129,131]. CD39 on endothelial cells and the vascular is highly expressed within pancreatic and rectal carcinoma. High

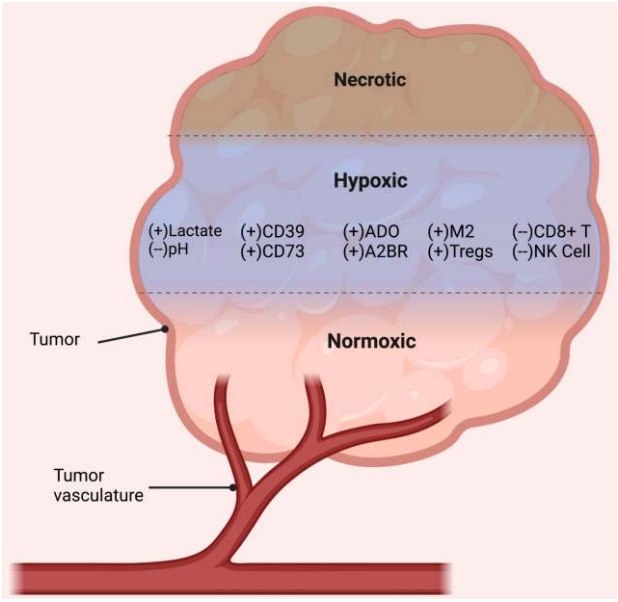

Figure 3. Hypoxia in the TME. This figure illustrates a region of a tumor devoid of vasculature, leading to an oxygen-starved environment. This hypoxic zone is characterized by low pH and a predominance of lactate. Within this environment, TAMs tend to adopt an M2 phenotype, and Tregs become the predominant T cell population. Hypoxic conditions also encourage the expression of the ectoenzymes CD39 and CD73, leading to a surge in extracellular ADO. This increase in adenosine, in turn, helps to sustain an immunosuppressive environment within the tumor. Note: this illustration does not depict the anatomical structure of the tumor, but rather represents the phenomena occurring at different levels of tumor oxygenation. ADO: Adenosine; A2BR: A2B receptors; NK: natural killer; TAM: tumor-associated macrophage; TME: tumor microenvironment; Tregs: regulatory T cells.

expression of these cells in these tumors is correlated with early TNM and better survival after tumor resection[132,133]. Studies have shown that having high levels of CD73 in many solid tumors is associated with worse outcomes. High CD73 levels correlate with higher adenosine concentrations in the tumor, leading to a sustained immunosuppressive TME. With a high expression of both CD39 and CD73 within solid tumors, future combination therapies targeting CD39/CD73, PD-1/CTLA4, and A2BR may allow for better survival in patients.

ADO receptors play various roles in cancer growth

An increase in adenosine within the TME allows for immunosuppression that promotes tumor growth. Receptors for ADO on tumor cells, endothelial cells, and immune cells are drivers of tumor growth and metastasis. The ADO G-protein coupled receptors have four subtypes: A1, A2A, A2B, and A3 [Figure 4]. The A1R, A2AR, and A2BR proteins are highly conserved, while the A3R varies among species. These receptors interact with MAPK pathways to promote proliferation. A2AR and A2BR also increase activation of the mTOR and ERK pathways[134]. However, receptor signaling is dependent on the concentration of extracellular ADO. This level of ADO is primarily dictated by ATP and ADP metabolism by CD39 and CD73 on cells to make AMP and then ADO. In the following, we will focus only on A2AR and A2BR, which appear to play major roles in tumor immunosuppression.

A2AR and A2BR increase immunosuppression in solid tumors

Both A2AR and A2BR are involved in ADO's immunosuppressive functions. A2AR and A2BR are expressed in most immune and tumor cells[59,135]. A2BR on myeloid cells normally has lower expression than A2AR on other immune cells. However, this receptor increases in the presence of pathological responses such as infections or cancer[136]. A2BR upregulation and activation signal macrophages towards the

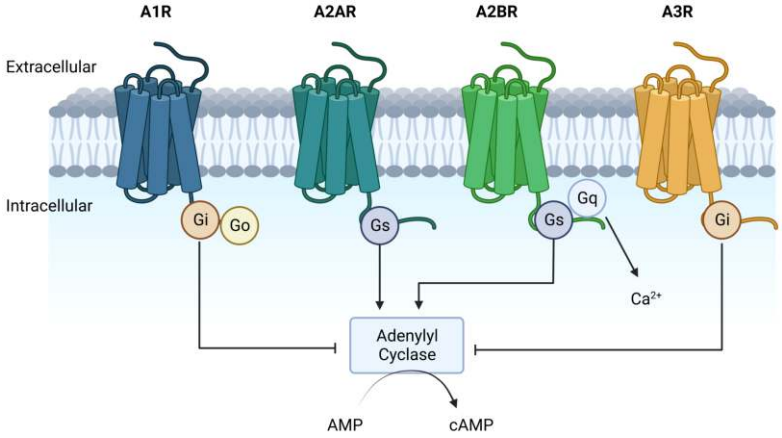

Figure 4. Adenosine receptors. Adenosine interacts with four distinct receptors - A1R, A2AR, A2BR, and A3R. Each of these receptors is linked to a G protein-coupled receptor. The A1R and A3R couple with the Gi protein to inhibit Adenylyl Cyclase. In contrast, the G protein coupled to A2AR and A2BR activates Adenylyl Cyclase, leading to an increased formation of intracellular cyclic AMP. A2BRs also couple to Gq which mobilizes Ca^{2+} upon activation. AMP: Adenosine monophosphate; A2AR: A2A receptors; A2BR: A2B receptors; cAMP: cyclic adenosine monophospha.

suppressive M2-like phenotype and secrete IL-10 and VEGF to promote angiogenesis and tumor growth[43]. A2ARs on infiltrating T cells within the TME are activated when ADO increases in the surroundings. This activation suppresses the effector CD8+ T cells in the TME while signaling CD4+ T cell differentiation into Tregs[137-139]. IFN-γ production decreases in NK cells when the A2AR on these cells is activated by ADO[99]. Decreases in antitumor cytotoxic cells and cytokines allow for increased suppressive factors that drive tumor growth and resistance to antitumor therapies.

A2BR activation due to TME hypoxia works to maintain the epithelial barrier of the tumor[39]. Maintaining this barrier prevents antitumor immune cells from penetrating the tumor. A2AR and A2BR-mediated immunosuppression also allow for an increase in metastasis amongst solid tumors[58].

A2BR is key for immunosuppression in solid tumors caused by ADO

Since A2BRs are expressed at low levels under normal conditions, this receptor may be key for triggering immunosuppression within solid tumors. The A2BR has the lowest potency for ADO under normal physiological conditions. However, during inflammation and sudden increases in apoptosis, the A2BR is activated to create an immunosuppressive niche. ADO drives immunosuppression in solid tumors by binding to the A2BR on immune and tumor cells. Once activated, the A2BR increases the secretion of VEGF and IL-8 into the TME[140,141]. This secretion from immune cells comes mainly from monocyte-derived immune cells. When activated by ADO and A2BR interactions, these cells contribute major driving factors in tumor immunosuppression. Ben Addi *et al.* discovered that the A2BR, not the A2AR, on bone marrow-derived DCs decreased the production of IL-12p70 in mice[84]. A2BR knock-out lung carcinoma cells produced lower VEGF levels in this model when stimulated with adenosine than wildtype controls[140].

Hypoxia increases A2BR expression

As described earlier, hypoxia is a hallmark of solid tumors. Recent studies have expanded our understanding of the role of hypoxia in solid tumors, emphasizing its impact on angiogenesis and tumor growth and its influence on other aspects of tumor biology, such as immune evasion and therapy resistance[18,142]. Hypoxia-driven upregulation of A2BRs and ADO signaling contributes to tumor progression, angiogenesis, and immune escape[44,113].

The hypoxic response also modulates the immune system, affecting innate and adaptive immunity. Hypoxia can alter the functions of immune cells such as TAMs, neutrophils, DCs, T cells, and NK cells[18,143]. Hypoxia-driven changes in the TME, for example, can create an immunosuppressive milieu, impairing the ability of immune cells to target and eliminate cancer cells[144]. Novel therapeutic strategies targeting hypoxia and adenosine signaling pathways, including A2BRs, are currently being investigated to improve the efficacy of existing cancer treatments and overcome treatment resistance[14].

The role of ADO and its receptors blockade to overcome resistance

ADO and its receptors are critical in maintaining immunosuppression in the TME, contributing significantly to immunotherapy resistance. Several clinical trials are exploring the potential of ADO receptor blockade as a novel strategy to counteract this resistance.

Several A2AR antagonists are currently being explored in clinical trials [Table 1], and two of the more advanced therapies are discussed further as part of this review: Corvus Pharmaceutical's ciforadenant and AstraZeneca's AZD4635[145].

In a first-in-human Phase 1 dose-escalation study in patients with advanced refractory cancers (NCT02655822), ciforadenant (either monotherapy or in combination with atezolizumab) was administered to 502 patients. Of those, a cohort of 68 renal cell carcinoma (RCC) patients yielded clinical responses, including partial responses (PR) in 11% of patients treated with a combination of A2AR antagonists and anti-PD-L1 antibodies, and in 3% of patients treated with A2AR antagonists alone[146]. Further, tumor regression was observed in an additional 24% of patients, although the regression was not significant enough to be classified as PR by RECIST criteria. These findings are noteworthy, especially considering that the patients involved in the study were not only resistant to PD-1 blockade but were also deemed untreatable prior to the trial.

According to Michail Sitkovsky[147], the observed tumor regressions in patients with RCC, who were previously untreatable and refractory to PD-1 blockade, likely occurred in patients meeting specific criteria: their tumors were immunogenic, developed tumor-reactive effector T cells, retained a significant number of effector cells post-toxic cancer chemotherapies, and were protected by immunosuppressive extracellular ADO to A2AR signaling. Ciforadenant appears to have facilitated the invasion and tumor-rejecting functions of T and NK cells in these patients; however, the levels of antitumor immunity in responsive patients were not high enough to achieve a complete response. The major limitation appears to be the lack or low numbers of tumor-reactive T and NK cells in refractory patients, either due to the tumor's poor immunogenicity or past toxic chemotherapies.

A2AR antagonists are anticipated to be most efficacious in patients with sufficient aggressive, multifunctional tumor-reactive T cells. Without these cells, it could be expected that A2AR antagonists would only have antitumor effects when combined with cancer vaccines or T-cell transfers that increase the number of tumor-reactive T cells. Future treatments combining A2AR antagonism with adoptive cell transfer (ACT) are promising, especially for refractory patients, as ACT ensures the presence of sufficient T-cells and NK-cells in patients, enhancing the potential for A2AR antagonism as an immunotherapy.

These antagonists have been able to show, both *in vitro* and *in vivo*, that blocking the adenosine pathway at the A2AR increases cytotoxic T cells within the TME, increases cytokine production, and reverses T cell inhibition.

Table 1. List of current clinical trials evaluating A2AR and A2BR antagonists alone or in combination with cancer immunotherapies

Drugs	Combinations	Clinical trial information				
		Phase	Indications	Enrollment	NCT number	Completion date
EOS448 (A2AR antagonist)	· EOS-448, a small molecule, combined with pembrolizumab, an anti-PD-1 antibody · EOS-448 combined with inupadenant, an investigational adenosine A2AR antagonist · EOS-448 combined with dostarlimab, an anti-PD-1 antibody · Inupadenant combined with dostarlimab · EOS-448 combined with inupadenant and dostarlimab · EOS-448 combined with dostarlimab and standard-of-care chemotherapies in participants with NSCLC	1/2	Lung/H&N cancers/Melanoma	376	NCT05060432	2024-09
NIR178 (Taminadenant)	· DFF332, a small molecule targeted to HIF-2α · DFF332 in combination with everolimus, an mTOR inhibitor · DFF332 in combination with spartalizumab (an anti-PD-1 antibody) plus taminadenant (A2AR antagonist)	1	RCC	180	NCT04895748	2025-04
TT-10 (A2AR antagonist)	· TT-10, a small molecule, as a single agent	1/2	Prostate/NSCLC/RCC	90	NCT04969315	2025-08
ILB2109 (A2AR antagonist)	· ILB2109, a small molecule, as a single agent	1	Advanced solid tumor	48	NCT05278546	2024-01
AZD4635 (A2AR antagonist)	· AZD4635 as monotherapy · Combination with durvalumab · Combination with durvalumab plus oleclumab · Combination with docetaxel · Combination with either abiraterone acetate or enzalutamide	1	Solid tumor	313	NCT02740985	2021-04
AZD4635 (A2AR antagonist)	· AZD4635 with durvalumab, an anti-PDL-1 antibody · AZD4635 with oleclumab, an anti-CD73 antibody	2	Prostate tumor	59	NCT04089553	2023-04
CPI-444 (A2AR antagonist)	· CPI-444, a small molecule, in combination with ipilimumab, an anti-CTLA4 antibody · CPI-444 in combination with nivolumab, an anti-PD-1 antibody	1/2	RCC	15	NCT05501054	2026-11
CPI-444 (A2AR antagonist)	· CPI-444 (ciforadenant) as a single · Combination with atezolizumab, a PD-L1 inhibitor	1	RCC	502	NCT02655822	2021-07
PBF-1129 (A2BR antagonist)	· Combination of adenosine A2BR antagonist PBF-1129 (mAb) and nivolumab, an anti-PD-1 antibody	1	Metastatic NSCLC	30	NCT05234307	2025-12
	· PBF-1129 as a single agent	1	Metastatic NSCLC	18	NCT03274479	2023-12
M1069 (Dual A2AR/A2BR antagonist)	· M1069, a small molecule, as a single agent	1	Unresectable solid tumors	30	NCT05198349	2023-12
AB928 (Dual A2AR/A2BR antagonist)	· Combination of SRF617, an anti-CD39 antibody, etrumadenant (AB928), and zimberelimab (AB122), an anti-PD-1 antibody	2	Prostate cancer	15	NCT05177770	2023-04
TT-4 (A2BR antagonist)	· TT-4, a small molecule, as a single agent	1/2	GI cancers	69	NCT04976660	2023-09

Data obtained from ClinicalTrials.gov. A2AR: A2A receptors; A2BR: A2B receptors; CTLA4: T lymphocyte antigen 4; GI: gastrointestinal; H&N: Head & Neck; NSCLC: non-small cell lung cancer; RCC: renal cell carcinoma.

Another A2AR antagonist in development is AstraZeneca's AZD4635. In a monotherapy phase 1 trial (NCT02740985), observed adverse events included nausea, fatigue, and vomiting. In addition, one patient with colorectal cancer had sudden death 15 days after the last dose of AZD4635, which was considered treatment-related by the investigator. However, AZD4635 was well tolerated both as a monotherapy and in combination with durvalumab in all patients. There were patients with responses such as stable disease, partial response, and complete response and RNA analysis confirmed that in 5 of 7 patients, intertumoral adenosine signaling decreased. Four of these 7 patients also had increases in gene-expression signatures of cytolytic activity and IFN-γ signaling. These findings suggest that there were observable positive responses to the treatment in some patients.

A2BR antagonists are also under investigation in several clinical trials. Arcus' etrumadenant, a dual A2AR and A2BR antagonist, is being evaluated in several cancers and was recently discontinued in mCRPC due to a lack of efficacy (NCT05177770). Palofarma's PBF-1129, an A2BR antagonist, is being evaluated in metastatic NSCLC (NCT03274479) and EMD Serono's M1069 (NCT05198349), another A2AR and A2BR antagonist, is currently undergoing a first-in-human trial in patients with advanced malignancies. In addition, Portage is evaluating TT-4, an A2BR antagonist, as a single agent in gastrointestinal cancer (NCT04976660). Further investigation will likely provide more insights into the clinical potential of these promising strategies. Overall, compounds exhibiting the highest water solubility tend to possess increased bioavailability, making them more effective[148]. With the advancement in the development of ADO receptor antagonists, enhancing the solubility of these promising compounds while preserving their selectivity emerges as an avenue for improvement.

CONCLUSION

In the complex battleground of cancer, it is necessary to understand the adaptations tumors employ to resist therapies. This review has emphasized the role of ADO, a significant player in the TME, in driving immunosuppression and fostering cancer drug resistance. The importance of ADO and its receptors, particularly the A2AR and A2BR subtypes, in promoting an immune-escaping environment was thoroughly explored.

Current research endeavors focus on various approaches to counteract immunosuppression, including monoclonal antibodies against CD73 and the blockade of ADO receptors. Ongoing clinical trials investigate combinations of these approaches with existing therapies, aiming to stimulate immune responses and improve patient outcomes.

The results of ongoing clinical trials will inform new ways of overcoming cancer drug resistance. However, further research is required to understand and fully exploit ADO's pathway. Targeting ADO could improve cancer treatments, providing hope for patients who previously had limited treatment options. This underscores the importance of ongoing research in this area, aiming to improve the prognosis for all cancer patients.

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Authors' contributions

Made substantial contributions to the conception, outline, and writing: Remley VA, Dimastromatteo J
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Cancer Drug Resistance

Review

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Immune checkpoint inhibitors in breast cancer: development, mechanisms of resistance and potential management strategies

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Abstract

The use of immune checkpoint inhibitors (ICIs) has increased exponentially in the past decade, although its progress specifically for breast cancer has been modest. The first U.S. Food and Drug Administration approval for ICI in breast cancer came in 2019, eight years after the first-ever approval of an ICI. At present, current indications for ICIs are relevant only to a subset of patients with triple-negative breast cancer, or those displaying high microsatellite instability or deficiency in the mismatch repair protein pathway. With an increasing understanding of the limitations of using ICIs, which stem from breast cancer being innately poorly immunogenic, as well as the presence of various intrinsic and acquired resistance pathways, ongoing trials are evaluating different combination therapies to overcome these barriers. In this review, we aim to describe the development timeline of ICIs and resistance mechanisms limiting their utility, and summarise the available approaches and ongoing trials relevant to overcoming each resistance mechanism.

Keywords: Immunotherapy, immune checkpoint inhibitors, resistance mechanisms, breast cancer

INTRODUCTION

Breast cancer is the most common cancer worldwide, accounting for 12.5% of all new cancer cases globally, and is the leading cause of cancer mortality in women[1]. In the year 2020, an estimated 2.3 million female breast cancers were diagnosed globally, and about 685,000 women died from their disease[2]. This number is expected to grow to more than 3 million new cases diagnosed and 1 million deaths by the year 2040[2].

With advances in our understanding of cancer biology, immuno-oncology has become an area of great interest and extensive research. Cancer immunotherapy employs the use of cutting-edge technologies, including immune checkpoint inhibitors (ICIs) such as those targeting Programmed Cell Death Protein-1 (PD-1), Programmed Cell Death Ligand-1 (PD-L1), and Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4), and more recently, chimeric antigen receptor (CAR) T cell therapies. Other frontiers being pushed in the realms of immunotherapy include the use of cancer vaccines[3], for cancer prevention, such as vaccines for Human Papilloma Virus and Hepatitis B[4], as well as in cancer treatment, as in the case of Sipuleucel-T for prostate cancer[5].

Since the first U.S. Food and Drug Administration (FDA) approval of ipilimumab, a CTLA-4 monoclonal antibody, in 2011 for the treatment of metastatic melanoma[6], ICIs have transformed the treatment landscape across multiple tumour types[7]. There are now eleven FDA approvals for ICIs: two CTLA-4 inhibitors (ipilimumab, tremelimumab), five PD-1 inhibitors (pembrolizumab, nivolumab, cemiplimab, dostarlimab, retifanlimab), three PD-L1 inhibitors (atezolizumab, avelumab, durvalumab), and one lymphocyte-activation gene 3 (LAG-3) blocking antibody (retatlimab)[6-9]. However, amongst the numerous available approvals for ICIs, there are currently only two specific FDA approvals in the setting of breast cancer, both for pembrolizumab in the subgroup of triple-negative breast cancer (TNBC), given in combination with chemotherapy in the metastatic[10] and neoadjuvant[11] settings. Additional FDA approvals that are tumour agnostic and apply to breast cancer include pembrolizumab[12] and dostarlimab[13] in breast cancers displaying high microsatellite instability (MSI-H) or a deficiency in the mismatch repair protein (dMMR) pathway. While one of the hallmarks of treatment with ICIs is its durable response that translates to prolonged survival of these patients, admittedly, only a very small subset of patients benefit. In this review article, we will first describe the evolution of ICI in the TNBC subtype, focusing on its approved indications, before delving into the understanding of the resistance mechanisms towards ICIs, and how we can harness such knowledge to develop new combination strategies.

EVOLUTION OF ICIS IN BREAST CANCER

Monotherapy ICIs in TNBC

Evidence for the use of ICIs in breast cancer first came from single-agent immunotherapy trials in the metastatic setting, including the KEYNOTE-012 and KEYNOTE-086 studies. KEYNOTE-012 was a phase I study that aimed to evaluate the role of single-agent pembrolizumab in patients with various advanced solid tumours. In the cohort of TNBC who had progressed on a median of 2 lines of treatment, the objective response rate (ORR) was 18.5% and 6-month progression-free survival (PFS) was 24.4%, 6-month and 12-month overall survival (OS) were 66.7% and 43.1%, respectively[14]. The investigators observed that there was a suggestion of response with increasing expression of PD-L1, albeit within a small sample size (n = 32).

KEYNOTE-086 was designed specifically to look at the role of pembrolizumab monotherapy in patients with metastatic TNBC. This phase II multicohort study included all comers with ≥ 1 prior systemic treatment for metastatic disease regardless of PD-L1 status (Cohort A)[15], and also patients with no prior systemic treatment in the metastatic setting who had PD-L1 positive tumours defined as combined positive score (CPS) ≥ 1 based on the Dako PD-L1 IHC 22C3 platform (Cohort B)[16]. Comparing across cohorts,

there was a suggestion of improved ORR in less heavily pre-treated patients (ORR 21.4% *vs.* 5.3%) in Cohort B *vs.* all-comers in Cohort A. This was consistent with other similar phase 1 trials evaluating avelumab (JAVELIN study)[17] and atezolizumab (PCD4989g trial)[18] as monotherapy in metastatic TNBC, suggesting clinical benefit when used in earlier lines of treatment and PD-L1 expressing tumours.

A subsequent KEYNOTE-119 randomised phase III trial compared pembrolizumab monotherapy *vs.* single agent physicians' choice chemotherapy in patients who progressed on 1 or 2 prior lines of treatment for metastatic TNBC[19]. While the trial was negative for its primary endpoint of OS in all subgroups, there was a positive trend to survival benefit in patients with PD-L1 CPS ≥ 10 (12.7 *vs.* 11.6 months, HR 0.78; $P = 0.057$).

The limited efficacy of single-agent immunotherapy observed in breast cancer might be due to intrinsic tumour resistance due to its complex and enigmatic relationship with the immune system. Breast cancer was traditionally thought to be poorly immunogenic, also known as a "cold tumour". Immunogenicity, or the ability to elicit an antitumoural response by the body's immune system, is dependent upon the formation of neo-antigens that are derived from gene mutations, viral oncogenes alternative splicing, or gene rearrangement[20-22]. It is assessed by the antigenicity of a cancer, which in turn is evaluated by its mutagenicity[23]. One measure of the antigenicity of cancer is its mutational load or tumour mutational burden (TMB), which refers to the average number of somatic mutations per (Mb)[23,24]. Cancers like melanoma and lung cancer are known to be "hot tumours", as observed in a study by Chalmers *et al.* who reported their median TMB levels to be 13.5 mut/Mb and 7.2 mut/Mb, respectively[25]. In contrast, the TMB in breast cancer is generally much lower. In a study by Barroso-Sousa *et al.*[26] of 3,969 patients with breast cancer, the median TMB reported was 2.63 mut/Mb, while another Chinese study of 196 breast cancer patients demonstrated a higher median TMB of 4.03 mut/Mb[27]. Due to the poor efficacy observed with the use of single-agent immunotherapy treatment, further efforts were directed at exploring combination treatment.

Combining ICIs with chemotherapy in TNBC

The rationale for combination treatment with chemotherapy was that chemotherapeutic agents had been shown to have synergistic effects with ICIs by inducing immunogenic cell death, causing the release of tumour-associated neoantigens as well as its ability to stimulate immune surveillance[28,29]. Indeed, this has proven to be an effective strategy in several subgroups of TNBC.

The initial results of several phase I studies evaluating this combination in the setting of metastatic TNBC were promising, reporting response rates ranging between 23.4%-39%[30,31]. Several phase III trials confirmed these positive preliminary findings, leading to the first FDA-approved indication for an ICI for use in breast cancer treatment.

Atezolizumab

The first FDA accelerated approval of an ICI for breast cancer was with the anti-PD-L1 inhibitor atezolizumab, which was granted on 8 March 2019[32] based on the IMpassion 130 trial[33]. This phase III placebo-controlled randomised trial evaluated 902 patients with treatment naïve, unresectable locally advanced or metastatic TNBC. Patients were randomised to receive either atezolizumab or its placebo, in combination with albumin-bound paclitaxel (nab-paclitaxel). In patients whose tumours expressed PD-L1 based on the VENTANA PD-L1 SP142 assay, there was a significant median PFS benefit: 7.5 months in patients receiving atezolizumab *vs.* 5.0 months with placebo (HR 0.62, $P < 0.001$). The final approval of this combination was contingent upon the results of the IMpassion 131 trial evaluating atezolizumab with paclitaxel in TNBC in the same setting, which unfortunately failed to meet its primary endpoint of superior

PFS[34]. This led to Genentech voluntarily withdrawing the previously granted accelerated FDA approval for atezolizumab on 27 August 2021. Eventually, when the final OS was read out for the IMpassion 130 trial, the addition of atezolizumab to nab-paclitaxel failed to meet statistical significance, precluding further testing[35].

Pembrolizumab

Pembrolizumab is currently FDA-approved for use in TNBC in the first-line metastatic and neoadjuvant settings, both in combination with chemotherapy. It first received FDA approval on 13 November 2020 as combination therapy with chemotherapy for patients with unresectable locally-advanced or metastatic TNBC whose tumours have a PD-L1 CPS ≥ 10 based on the Dako 22C3 assay[10]. This was based on KEYNOTE-355, a phase III randomised placebo-controlled study evaluating the role of pembrolizumab in combination with chemotherapy in patients in the above-mentioned setting. It reported a median OS (mOS) benefit of about 7 months in patients whose tumours expressed PD-L1 CPS ≥ 10 (mOS 23.0 *vs.* 16.1 months; HR 0.73, $P = 0.0185$). In patients whose tumours expressed PD-L1 CPS ≥ 1 or in the intention-to-treat population, there was no survival benefit shown.

In addition, pembrolizumab also has tumour-agnostic FDA approval for advanced unresectable or metastatic solid tumours that are dMMR or MSI-H[12]. This was based on the combined results of 5 single-arm trials where a total of 149 patients with dMMR/MSH-H solid tumours achieved an ORR of 39.6%, with 78% of patients having responses lasting 6 months or more. It should be noted, however, that only 2 out of the 149 patients had breast cancer. They both achieved partial responses, with duration of response (DoR) of 7.6 and 15.9 months[36].

Dostarlimab

Most recently, on 17 August 2021, dostarlimab also received accelerated FDA approval for recurrent or advanced solid tumours that are dMMR based on the GARNET trial[13]. This was an open-label, non-randomised, multicohort phase I trial evaluating dostarlimab as monotherapy in the above-mentioned clinical setting. In these patients, there was an ORR of 41.6%, with 9.1% complete responses and 32.5% partial responses. The median DoR was 34.7 months, with 95.4% of patients still showing continued response at 6 months. In cohort F, which enrolled 106 non-endometrial solid tumours, 1 patient had dMMR breast cancer and reported a complete response[37].

With the promising results of a combination of ICI therapy and chemotherapy in the metastatic setting, efforts were then shifted to study it in the earlier curative stages of breast cancer. One of these trials is the phase II I-SPY 2 trial, which adopted an adaptive trial design to evaluate various novel therapeutics in combination with chemotherapy, comparing that to standard treatment as in the neoadjuvant setting for early-stage breast cancer[38]. Pembrolizumab was included in one of the study arms, where patients were randomised to receive 4 cycles of pembrolizumab given in combination with weekly paclitaxel *vs.* weekly paclitaxel alone, followed by doxorubicin/cyclophosphamide and then definitive surgery. Compared to standard chemotherapy alone, the addition of pembrolizumab improved pathologic complete response (pCR) rates in all breast cancer subtypes: 44% *vs.* 17% in HER2-negative breast cancers, 30% *vs.* 13% in HR-positive/HER2-negative breast cancers, and 60% *vs.* 22% in TNBC[39].

Focusing on the TNBC subtype, the role of pembrolizumab in the neoadjuvant setting was proven in the confirmatory phase III KEYNOTE-522 trial, which subsequently led to pembrolizumab receiving its second breast cancer-specific FDA approval on 26 July 2021[11]. In this phase III randomised controlled study, 1,174 patients with previously untreated stage II or III TNBC were randomised in a 2:1 ratio to receive pembrolizumab or a placebo, respectively, in combination with chemotherapy , before undergoing surgery.

Pembrolizumab or its placebo was continued post-operatively for up to 9 cycles. Both primary endpoints of the trial were met; there was a significant improvement in pCR of 64.8% *vs.* 51.2%; $P = 0.00055$, although this had reduced by the third interim analysis[40] to 63.0% *vs.* 55.6%. There was also an improvement in 3-year event-free survival (EFS) 84.5% *vs.* 76.8%; $P < 0.001$[41]. Interestingly, contrary to data in the metastatic setting, PD-L1 expression was not predictive of benefit[11], and consequently the FDA approval in the neoadjuvant setting was granted irrespective of PD-L1 expression.

The benefit of ICIs in combination with chemotherapy in the neoadjuvant setting was also echoed in the IMpassion 031 study evaluating atezolizumab. In IMpassion 031, atezolizumab was evaluated in the neoadjuvant setting in patients with stage II-III TNBC treated for curative intent. This was a double-blind phase III randomised trial where patients received either atezolizumab or its placebo, in combination with nab-paclitaxel, followed by doxorubicin and cyclophosphamide. The investigators found an increase in pCR rates from 58% *vs.* 41% in the all-comers population; $P = 0.0044$, (significance boundary 0.0184), and 69% *vs.* 49% in PD-L1 positive patients; $P = 0.021$, (significance boundary 0.0184). As it did not hit the prespecified boundary of significance for its second co-primary endpoint, the study is not formally powered for further survival analyses[42].

However, not all trials evaluating the addition of ICIs in combination with chemotherapy in the neoadjuvant setting for TNBC have yielded similar results. Both the NeoTRIP and GeparNeuvo evaluating atezolizumab and durvalumab, respectively, in the neoadjuvant setting were negative for pCR benefit. Patients in the NeoTRIP study were randomised to receive neoadjuvant carboplatin and nab-paclitaxel with or without 8 cycles of atezolizumab. Anthracyclines were given in the adjuvant setting after definitive surgery. The addition of atezolizumab resulted in numerically higher but nonsignificant pCR rates: 48.6% *vs.* 44.4%; $P = 0.48$[43]. Similarly, the GeparNuevo trial studied the addition of durvalumab to neoadjuvant chemotherapy with paclitaxel followed by epirubicin and cyclophosphamide, which found a nonsignificant but numerically superior pCR rates of 53.4% *vs.* 44.2%; $P = 0.224$[44]. Interestingly, a survival benefit with the addition of durvalumab compared to placebo was observed; 3-year invasive disease-free survival (iDFS) was 84.9% *vs.* 76.9% (HR 0.54; $P = 0.0559$) and 3-year OS 95.1% *vs.* 83.1% (HR 0.26; $P = 0.0076$)[45].

While there is general consensus for the use of ICIs in combination with chemotherapy in the neoadjuvant setting for TNBC, its optimal duration is currently still widely discussed. In both KEYNOTE-522 and IMpassion 031, the ICI was continued post-operatively for a total of 1 year, while NeoTRIP and GeparNuevo only administered ICI in the neoadjuvant setting. GeparNuevo is the only study that has shown survival benefits with the use of ICI despite being administered only in the neoadjuvant context without continuation in the adjuvant setting, leading to questions of whether there is a need for continual ICI in the adjuvant setting. Additionally, the pCR benefit that was observed in the durvalumab group in GeparNuevo was exclusively seen in the cohort of patients who received a 2-week lead-in of durvalumab prior to chemotherapy, although the reason for this observation is currently unclear. We have summarised the trials evaluating the use of ICI both as monotherapy and in combination with chemotherapy in Table 1.

In the adjuvant setting, there are ongoing trials such as the A-BRAVE trial[46] investigating the use of avelumab in the treatment of high-risk TNBC, as well as the ALEXANDRA/IMpassion030 trial[47] evaluating standard chemotherapy with or without atezolizumab in patients with early-stage TNBC. Additionally, the use of ICIs in early relapsing TNBC is also being investigated in the IMpassion 132 trial, a phase III randomised trial evaluating the role of combining atezolizumab with chemotherapy in patients with locally recurrent inoperable or metastatic TNBC within 12 months from receiving curative-intent treatment[48].

Table 1. Summary of trials evaluating the use of ICI as monotherapy and in combination with chemotherapy

Trial name/ID	Phase	Population	Arms	Results
KEYNOTE-012 NCT01848834	I	Advanced TNBC, PD-L1 + ve; pre-treated	Pembrolizumab	ORR 18.5% 6-mo PFS 24.4% 6-mo OS 66.7%, 12-mo OS 43.1%
KEYNOTE-086 NCT02447003	II	Metastatic TNBC; pre-treated Cohort A: all-comers Cohort B: PD-L1 + ve	Pembrolizumab	Cohort A: ORR 5.3%, mPFS 2.0 mo, mOS 9.0 mo Cohort B: ORR 21.4%, mPFS 2.1 mo, mOS 18.0 mo
JAVELIN NCT01772004	I	Metastatic breast cancer; pre-treated	Avelumab	ORR: 3.0% (overall population), 5.2% (TNBC), 16.7% (PD-L1 + ve), 1.6% (PD-L1-ve)
PCD4989g NCT01375842	I	Metastatic TNBC; any-line	Atezolizumab	ORR 24% (1st line), 6% (≥ 2nd line) ORR 12% (1st line), 0% (≥ 2nd line) mOS 10.1 mo (PD-L1 + ve), 6.0 mo (PD-L1-ve)
KEYNOTE-119 NCT02555657	III	Metastatic TNBC; 1 or 2 prior lines	Pembrolizumab *vs.* chemotherapy	mOS 9.9 mo *vs.* 10.8 mo HR 0.97 (overall population) mOS 12.7 mo *vs.* 11.6 mo HR 0.78; *P* = 0.057 (PD-L1 CPS ≥ 10)
IMpassion 130 NCT02425891	III	Metastatic TNBC; untreated	Nab-paclitaxel +/- atezolizumab	mPFS 7.2 mo *vs.* 5.5 mo, HR 0.79; *P* = 0.002 (ITT) mOS 21.0 mo *vs.* 18.7 mo HR 0.87; *P* = 0.077 (ITT) mPFS 7.5 mo *vs.* 5.0 mo HR 0.63, *P* < 0.0001 (PD-L1 + ve) mOS 25.4 mo *vs.* 17.9 mo HR 0.67; (PD-L1 +ve)
IMpassion 131 NCT03125902	III	Metastatic TNBC; untreated	Paclitaxel +/- atezolizumab	mPFS 6.0 mo *vs.* 5.7 mo, HR 0.82; *P* = 0.20 (PD-L1 + ve) mPFS 5.7 mo *vs.* 5.6 mo, HR 0.86 (ITT)
KEYNOTE-355 NCT02819518	III	Metastatic TNBC; untreated	Chemotherapy +/- pembrolizumab	mPFS 9.7 mo *vs.* 5.6 mo HR 0.66 (CPS ≥ 10) mPFS 7.6 mo *vs.* 5.6 mo HR 0.75 mOS 23.0 *vs.* 16.1 mo HR 0.73; *P* = 0.0185 (CPS ≥ 10) mOS 17.6 mo *vs.* 16.0 mo HR 0.86 *P* = 0.1125 (CPS ≥ 1)
I-SPY 2 NCT01042379	II	High-risk stage II/III breast cancer	Chemotherapy +/- pembrolizumab	pCR 44% *vs.* 17% (ERBB2-negative), 30% *vs.* 13% (HR- + ve/ERBB2-ve), 60% *vs.* 22% (TNBC)
KEYNOTE-522 NCT03036488	III	Stage II/III TNBC	Chemotherapy +/- pembrolizumab	pCR 64.8% *vs.* 51.2%; *P* = 0.00055 3yr EFS 84.5% *vs.* 76.8% HR 0.63; *P* < 0.001
IMpassion-031 NCT03197935	III	Stage II/III TNBC	Chemotherapy +/- pembrolizumab	pCR 58% *vs.* 41%; *P* = 0.0044 (all-comers) pCR 69% *vs.* 49% *P* = 0.021 (significance boundary 0.0184) (PD-L1 + ve)
NeoTRIP NCT002620280	III	Early high-risk and locally advanced TNBC	Chemotherapy +/- atezolizumab followed by surgery, then adjuvant anthracyclines	pCR 48.6% *vs.* 44.4% OR 1.18; *P* = 0.48
GeparNuevo NCT02685059	II	Non-metastatic TNBC	Chemotherapy +/- durvalumab *window phase included 2 weeks of durvalumab/placebo	pCR 53.4% *vs.* 44.2% OR 1.45; *P* = 0.224 3yr iDFS 84.9% *vs.* 76.9% HR 0.54; *P* = 0.0559 3yr OS 95.1% *vs.* 83.1% HR 0.26; *P* = 0.0076

CPS: Combined positive score; EFS: event-free survival; ICI: immune checkpoint inhibitor; iDFS: invasive disease-free survival; mOS: median OS; ORR: objective response rate; OS: overall survival; pCR: pathologic complete response; PD-L1: programmed cell death ligand-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

ICIs in other subtypes of breast cancer

While there have also been efforts to evaluate the use of ICIs in HER2-positive and hormone-positive/HER2-negative breast cancers, none of the studies have led to conclusive evidence for its use in these settings at present. In particular, HER2-positive breast cancer is thought to share certain similarities with TNBC that might suggest a benefit from ICI therapy. This includes the presence of higher tumour infiltrating lymphocytes (TILs) and PD-L1 expression. The presence of TILs in the tumour and its

surrounding microenvironment is thought to be a reflection of pre-existing antitumour immunity[49,50], and its presence is thought to be predictive of response to systemic anti-cancer treatment[50], as well as a prognostic biomarker[24]. TNBC and HER2-positive breast cancers have been observed to have a higher number of TILs compared to hormone-positive breast cancers[51,52]. PD-L1 expression has also been observed to be upregulated in HER2-positive breast cancer[53], and be predictive of response to ICIs in the PANACEA and KATE2 studies[54,55]. Further in-depth discussion of ICIs in HER2-positive and hormone-positive/HER2-negative breast cancers is beyond the scope of our current article, but has been extensively reviewed[56-58].

UNDERSTANDING AND OVERCOMING RESISTANCE MECHANISMS TO ICIS

Given that the earliest approval for ICI use in breast cancer came on 8 March 2019 for atezolizumab in combination with nab-paclitaxel in metastatic TNBC based on the IMpassion 130 trial[33], the experience and evidence available on resistance mechanisms specific to immunotherapy in breast cancer is scarce. In addition, discounting tumour agnostic approvals, which form a very small proportion of breast cancer patients as discussed above[36,37], the approval for ICIs in breast cancer is now only limited to the TNBC subtype, which constitutes only 15%-20% of all patients with breast cancer[59], and even so, only a subset of them with high risk early-stage and metastatic disease. Hence, much of our understanding of resistance to ICIs comes from the available data and research on ICI treatment as a whole from various other tumour types.

Resistance pathways to ICIs can be tumour-intrinsic, e.g., alteration of certain genes or signalling pathways within the tumour, or tumour-extrinsic, e.g., changes in components within the tumour microenvironment (TME) other than the tumour cell itself[60]. This can happen either from the outset, conferring primary resistance whereby no response to treatment is noted, or after a period of observed response, highlighting the concept of acquired resistance. As previously mentioned, breast cancers are known to be immunogenically cold tumours, which contributes to their primary resistance to ICI. We will discuss the various mechanisms of resistance by looking at both tumour-intrinsic and tumour-extrinsic pathways, and how each of them might potentially be harnessed to overcome drug resistance [Figure 1].

TUMOUR INTRINSIC RESISTANCE MECHANISMS TO ICIS

Alteration of signalling pathways

There are several critical signalling pathways that control cell-cycle progression, apoptosis, and cell growth. Alterations in any of these pathways can sometimes be exploited by cancer cells to escape immune surveillance, leading to resistance to ICIs. Some of these pathways are known to be more commonly mutated in breast cancer, for example, the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, Wnt/β-catenin pathway and Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathway[61]. Hence, various combination therapies of ICIs with other therapeutic agents to target each of these specific pathways are gaining traction and have shown promising preliminary activity.

MAPK pathway

Signalling via the MAPK pathway induces the expression of various proteins such as vascular endothelial growth factor (VEGF) as well as interleukin (IL)-8 that inhibit T cell recruitment and function[62]. Inhibiting the MAPK pathway can also upregulate major histocompatibility complex (MHC)-I, MHC-II, and PD-L1 expression, and enhance infiltration of TILs[63]. Loi *et al.* had confirmed this observation in an analysis of 111 patients with TNBC who had been treated with neoadjuvant chemotherapy, and demonstrated that

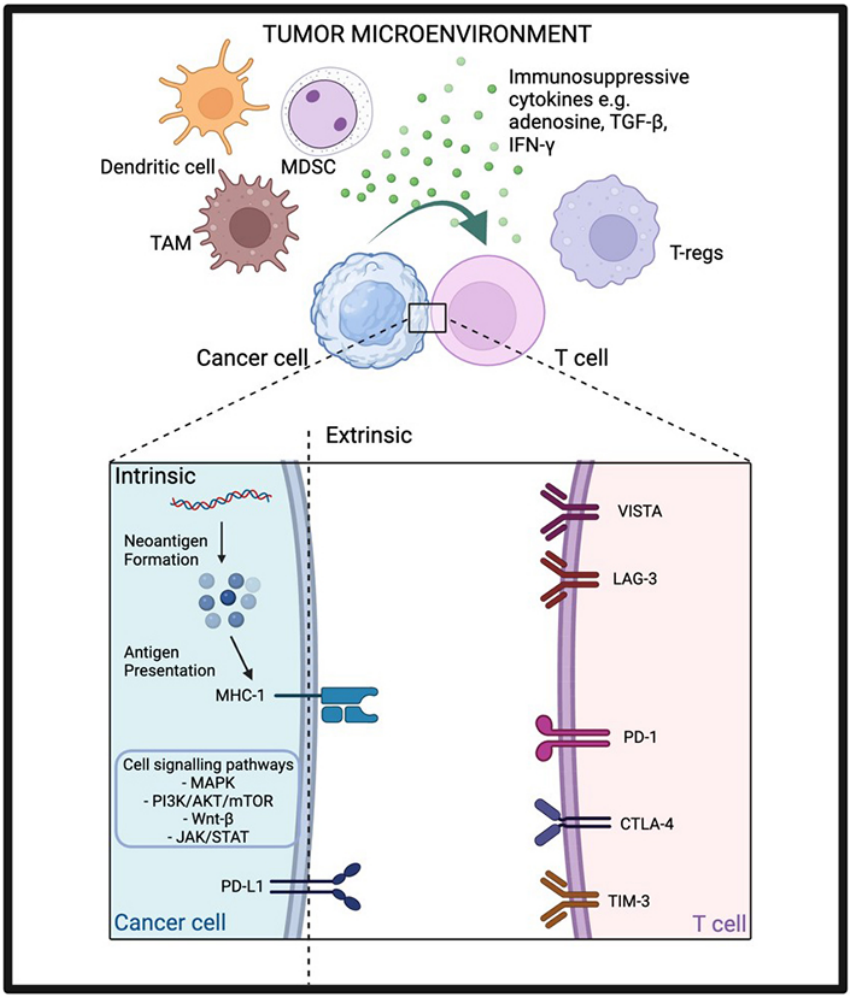

Figure 1. Tumour intrinsic and extrinsic resistance pathways to ICIs. Created with BioRender.com. AKT: Protein kinase B; CTLA-4: cytotoxic T-lymphocyte-associated antigen 4; IFN-γ: interferon-γ; JAK: janus kinase; LAG-3: lymphocyte-activation gene 3; MAPK: mitogen-activated protein kinase; MDSC: myeloid-derived suppressor cell; MHC-I: major histocompatibility complex-I; mTOR: mammalian target of rapamycin; PD-1: programmed cell death protein-1; PD-L1: programmed cell death ligand-1; PI3K: phosphatidylinositol 3-kinase; STAT: signal transducers and activators of transcription; TAM: tumour-associated macrophage; TGF-β: transforming growth factor-β; TIM-3: T-cell immunoglobulin, mucin domain-3 protein; T-reg: regulatory T cell; VISTA: V-domain immunoglobulin suppressor of T-cell activation.

alterations in the MAPK signalling pathway can suppress the expression of both MHC-I and MHC-II[64].

Hence, trials evaluating the combination of mitogen-activated protein kinase kinase (MEK or MAP2K) inhibitors with ICIs are ongoing. The COLET trial[65] was a phase II trial that investigated cobimetinib, a MEK inhibitor, in combination with atezolizumab and taxane chemotherapy in untreated metastatic TNBC. It demonstrated a numerical but nonsignificant increase in ORR of 34.4% and 29% in patients treated with paclitaxel *vs.* nab-paclitaxel, respectively. Exploratory biomarker analysis suggested that patients with PD-L1-positive disease (defined as IC ≥ 1% by the SP142 IHC assay) had numerically higher ORR compared to those with PD-L1 negative disease (39% *vs.* 19%), as well as median PFS (7.0 *vs.* 3.7 months).

PI3K/AKT/mTOR pathway

Abnormalities in the PI3K/AKT/mTOR pathway are also another well-known mechanism of resistance in breast cancer[66]. The protein phosphatase and tensin homolog (PTEN) tumour suppressor is a negative regulator of PI3K signalling and deletions in PTEN result in the enhancement of PI3K signalling[66,67]. PTEN loss has also been associated with resistance to T cell-mediated immunotherapy by increasing the expression of immunosuppressive cytokines, particularly VEGF[68]. VEGF can contribute further to immunosuppressive TME by recruiting suppressive immune cells such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs)[69].

Based on these preclinical findings, AKT inhibitors have been combined with ICIs to overcome this resistance pathway. The phase Ib study evaluating the triplet combination of ipatasertib, atezolizumab, and a taxane as first-line treatment for locally advanced/metastatic TNBC reported a promising ORR of 73% irrespective of their PD-L1 status or PIK3CA/AKT1/PTEN alteration status[70]. The BEGONIA study (NCT03742102) is a phase Ib/II trial evaluating the combination of durvalumab with different novel oncologic therapies designed for immune modulation, with or without paclitaxel as first-line treatment in patients with metastatic TNBC. In arm 2[71], the addition of capivasertib was studied, yielding an ORR of 53.3%. Importantly, there was a relatively high rate of G3/4 treatment-related adverse events of 73%, although only 6.7% discontinued treatment due to adverse events.

Wnt/β-catenin pathway

The Wnt/β-catenin pathway is an important oncogenic signalling pathway involved in many essential cellular processes[72]. The activation of Wnt results in the accumulation of the transcriptional co-activator β-catenin to initiate the transcription of several cell cycle genes and oncogenes such as Myc[73]. The high levels of β-catenin via the canonical pathway have also been shown in a murine study by Spranger *et al.* to decrease the presence of CD103+ dendritic cell (DC) by reducing the expression of chemokine that attracts CD103+ DC (CCL4), preventing the migration of DC into the TME[74]. Consequently, this results in the blocking of adaptive antitumour immunity[75]. A study of TNBC by Castagnoli *et al.* showed that TNBC stem cells are able to upregulate PD-L1 expression via the Wnt pathway[75].

JAK/STAT pathway

Interferon γ (IFN-γ) is a cytokine produced by activated T cells and antigen-presenting cells (APCs) that is critical in immune cell activation via the Janus kinase 1 and 2 (JAK1/2) as well as signal transducers and activators of transcription-1 (STAT1) pathway[76]. Any mutation or epigenetic silencing of molecules in this pathway allows tumours to escape its apoptotic or cytostatic effect[77]. A study analysing melanoma patients who were treated with ICI therapy and subsequently developed resistance noted that resistance was associated with defects such as loss-of-function mutations in the JAK1/2 pathway[78]. Another study of 16 melanoma patients observed that those who were non-responders to CTLA-4 inhibition harbor a much higher rate of genomic changes in the IFN-γ pathway genes compared to those who responded[79].

Antigen presentation

A crucial feature of adaptive immunity is its ability to recognise antigens that are foreign or not "self". Cancer cells generally harbour accumulated somatic mutations and genomic instability within DNA coding regions. Antigen peptide sequences that distinguish tumour cells are classified based on their unique cell expression patterns[73]. Tumour-specific antigens (TSA) refer to novel peptide sequences, i.e., neo-antigens, that develop via mutations and are not present in normal healthy cells. Examples of mutations that result in TSAs usually involve oncogenic driver mutations, such as mutations in the *BRCA1/2* gene[80]. The presentation of these neoantigens by APCs via MHC-I molecules is critical in priming specific cytotoxic

CD8+ T cells, thereby triggering an immune response towards the tumour. Indeed, studies have shown that increasing neoantigen formation helps to improve response to ICIs[81-83].

Antibody-drug conjugates

Similar to the rationale for combining ICIs with chemotherapy which was discussed earlier, antibody-drug conjugates (ADCs) also increase tumour neoantigen formation via immunogenic cell death[84]. An ADC consists of an antigen-specific monoclonal antibody bound to a cytotoxic payload via a molecular linker. The binding of an ADC via its antigen-binding portion induces its internalisation via endocytosis. Once inside the tumour cell, cleavage of its linker through proteolysis results in the release of the cytotoxic payload. This allows for target-dependent activation and selective cytotoxicity[85]. Of note, two ADCs, namely trastuzumab deruxtecan (T-DXd) and sacituzumab govitecan, have received FDA approvals for the treatment of specific breast cancer subtypes. T-DXd is approved for unresectable or metastatic HER2-positive breast cancer based on the results of DESTINY-Breast 03, confirming significant PFS benefit (HR 0.28; $P < 0.0001$)[86], as well as for unresectable or metastatic HER2-low breast cancer based on DESTINY-Breast 04 showing both promising PFS (HR 0.50; $P < 0.001$) and OS (HR 0.64; $P = 0.001$) benefit[87]. Sacituzumab govitecan, on the other hand, has been approved both for unresectable or metastatic TNBC as well as hormone-positive, HER2-negative breast cancer based on the ASCENT and TROPiCS-02 trials, respectively, both confirming PFS and OS benefit[88,89].

Preclinical data have suggested that the combination of ADCs with ICIs may improve the efficacy of ICIs via increasing neoantigen formation and presentation, as well as by activating DCs and increasing the expression of PD-L1[85]. There are currently several ongoing trials evaluating the combination of different ADCs with ICIs. In the earlier described BEGONIA study, two ADCs, T-DXd and datopotamab deruxtecan (Dato-DXd), are being studied in arms 6 and 7 of the trial, respectively. Preliminary data for both arms were promising; ORR with the addition of T-DXd was 66.7%[90] and 74% with the addition of Dato-DXd[91]. Other ongoing trials in this space are summarised in Table 2.

Poly(ADP-ribose) polymerase inhibitors

Poly(ADP-ribose) polymerase (PARP) inhibitors increase DNA damage, leading to more TSAs and also increased MHC-I expression, thereby causing increased antigen presentation[92]. The increase in DNA damage associated with breast cancer patients who harbour the *BRCA1/2* mutation occurs via a process known as synthetic lethality[93]. The use of PARP inhibitors blocks the repair of single-stranded DNA breaks via base excision repair. This allows single-stranded breaks to accumulate, leading to the generation of double-stranded breaks (DSBs). These DSBs can usually be restored by either the high-fidelity homologous repair pathway or the error-prone non-homologous end-joining method. As *BRCA1/2* mutant breast cancer patients already have existing defects in homologous repair, they are unable to effectively repair DNA damage, resulting in the generation of TSAs. In addition to increasing antigen presentation, PARP inhibitors have also been shown in preclinical studies to alter the TME by activating intra-tumoural dendritic cells and increasing CD8+ T cell infiltration via the STING (stimulator of interferon genes) pathway[94]. It also enhances the upregulation of PD-L1 expression by reducing the PARylation of STAT3[95]. The latter two mechanisms help to overcome tumour extrinsic mechanisms of resistance to immunotherapy that will be expounded upon later.

Consequently, there have been several studies evaluating the combination of PARPi together with ICIs. The TOPACIO/KEYNOTE-162 trial[96] studied the efficacy of niraparib together with pembrolizumab in 55 patients with metastatic TNBC. In the subgroup of patients with *BRCA1/2* mutations, the ORR was 47% and mPFS 8.3 months. In contrast, patients who were non-*BRCA1/2* mutants had an ORR of 11% and mPFS of

Table 2. Summary of ongoing trials evaluating the addition of ADC to ICI therapy

Trial name/ID	Phase	Patients enrolled	ICI	ADC	Primary endpoint(s)
ASCENT-04 NCT05382286	III	Treatment naïve advanced/metastatic TNBC	Pembrolizumab	Sacituzumab Govitecan	PFS
NCT04448886	II	Metastatic HR+/HER2- breast cancer who have progressed on or within 12 months of adjuvant endocrine or ≥ 1 endocrine therapy in the metastatic setting	Pembrolizumab	Sacituzumab Govitecan	PFS
NCT03310957	I/II	Advanced/Metastatic TNBC	Pembrolizumab	SGN-LIV1A	ORR, DLT, adverse events
Morpheus-TNBC NCT03424005	Ib/II	Metastatic TNBC	Atezolizumab	Sacituzumab Govitecan or SGN-LIV1A	ORR, adverse events
InCITe NCT03971409	II	Metastatic TNBC	Avelumab	Sacituzumab Govitecan	ORR
Astefania NCT04873362	III	Patients with residual invasive disease in breast/axillary lymph nodes following neoadjuvant chemotherapy	Atezolizumab	Trastuzumab emtansine	Invasive disease-free survival
KATE3 NCT04740918	III	Metastatic PD-L1-positive cancer after progression on H +/- P and taxane	Atezolizumab	Trastuzumab emtansine	PFS, OS
NCT03032107	I	Metastatic breast cancer on progression on prior H and a taxane	Pembrolizumab	Trastuzumab emtansine	Safety and tolerability
NCT04042701	Ib	Metastatic HER2 positive or HER2 low breast cancer	Pembrolizumab	Trastuzumab deruxtecan	DLT and ORR
NCT03523572	I	Metastatic breast cancer progressed on ≥ 2 anti-HER2-based regimens	Nivolumab	Trastuzumab deruxtecan	DLT, ORR
DESTINY-Breast07 NCT04538742	Ib/II	Metastatic 2nd line and beyond (Part 1) and 1st line (Part 2)	Durvalumab	Trastuzumab deruxtecan	Safety and toxicity
DESTINY-Breast08 NCT04556773	I	Advanced or metastatic HER2-low breast cancer	Durvalumab	Trastuzumab deruxtecan	Safety and toxicity

ADC: Antibody-drug conjugate; DLT: dose-limiting toxicities; ICI: immune checkpoint inhibitor; ORR: objective response rate; OS: overall survival; PD-L1: programmed cell death ligand-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

2.1 months. The MEDIOLA trial[97] studied the combination of olaparib and durvalumab as first or second-line therapy in germline *BRCA1/2* mutant metastatic TNBC, noting an ORR of 63%, mPFS of 8.2 months and mOS 21.5 months. Table 3[96-101] summarises some of the available trials evaluating this combination.

Tumour cells can also evade immune surveillance by altering any step in the antigen presentation pathway, thereby conferring resistance to treatment with ICIs. Several studies involving patients with breast cancer have reported the downregulation of expression of the transporters TAP1, TAP2, and TAPBP, which are necessary for transporting antigens to be loaded onto MHC molecules[102-104]. Other mechanisms that have been observed include loss of heterozygosity and epigenetic suppression of certain MHC-I molecules[105] or alterations in the expression of beta-2-microglobulin (B2M) which is essential for the transport and subsequent expression of MHC-I on the cell surface[105,106]. Luo *et al.* reported the potential use of DNA methyltransferase inhibitors to overcome resistance to immunotherapy in breast cancer patients[107].

TUMOUR EXTRINSIC MECHANISMS OF RESISTANCE TO IMMUNOTHERAPY

Alteration of the tumour microenvironment

The TME comprises various components that are constantly evolving, with ongoing crosstalk between tumour and stromal cells, all of which can influence the immune response and drive resistance to ICIs[73]. The presence of TILs in the tumour and its surrounding microenvironment is thought to be a reflection of pre-existing antitumour immunity[49,50], and its presence is thought to be predictive of response to systemic anti-cancer treatment[50], and a prognostic biomarker[24]. TNBC and HER2-positive breast cancers have a

Table 3. Summary of ongoing trials evaluating the addition of PARPi to ICI therapy

Trial name/ID	Phase	Patients enrolled	ICI	PARPi	Primary endpoint(s)	Results (if any)
TOPACIO/ KEYNOTE-162 NCT02657889	I/II	Advanced or metastatic TNBC	Pembrolizumab	Niraparib	DLT and ORR	ORR 21%, 47%, 11% (overall, BRCA mutant, BRCA wild-type)[96]
NCT04683679	II	Metastatic TNBC or HR+/HER2- breast cancer	Pembrolizumab	Olaparib	ORR	
NCT03101280	Ib	Previously treated metastatic TNBC with BRCA mutation or BRCA-like molecular signature	Atezolizumab	Rucaparib	Number of dose modifications due to adverse events	
NCT02849496	II	Advanced or metastatic non-HER2-positive breast cancer with homologous DNA repair deficiency	Atezolizumab	Olaparib	PFS	
NCT04690855	II	Germline BRCA1/2 negative, PD-L1 positive metastatic TNBC	Atezolizumab	Talazoparib	ORR	
MEDIOLA NCT02734004	I/II	Germline BRCA mutated metastatic HER2-negative breast cancer	Durvalumab	Olaparib	DCR, safety, and tolerability	DCR at 12 weeks 80%, 28 weeks 50% ORR 63.3%[97]
DORA NCT03167619	II	Platinum-treated metastatic TNBC	Durvalumab	Olaparib	PFS	Combination arm: mPFS 6.1 mo, DCR 68.2%[98]
DOLAF NCT04053322	II	Advanced ER+, HER2- breast cancer with BRCA mutation, alteration in homologous recombination repair or MSI	Durvalumab	Olaparib	PFS	
PHOENIX NCT03740893	II	Post-neoadjuvant chemotherapy with residual TNBC	Durvalumab	Olaparib	Biomarker study pre-surgery and post-surgery	
NCT03801369	II	Metastatic TNBC	Durvalumab	Olaparib	ORR	
NCT03544125	I	Metastatic TNBC	Durvalumab	Olaparib	Safety and efficacy	
NCT02484404	I/II	Advanced TNBC	Durvalumab	Olaparib	Dose finding and toxicities	
JAVELIN PARP Medley NCT03330405	Ib/II	Advanced/ metastatic TNBC or HR+/HER2- breast cancer	Avelumab	Talazoparib	DLT and ORR	ORR 18.2% and 34.8% (TNBC, HR+/HER2-)[99]
JAVELIN BRCA/ATM NCT03565991	II	BRCA or ATM mutant advanced or metastatic solid tumour	Avelumab	Talazoparib	ORR	ORR 26.4% (BRCA) 4.9% (ATM)[100]
TALAVE NCT03964532	I/II	Advanced breast cancer	Avelumab	Talazoparib	Safety and toxicities	
NCT03945604	Ib	Recurrent, metastatic TNBC	Camrelizumab (anti-PD-1)	Fluzoparib	DLT	mPFS 5.2 mo, 12 mo OS 64.2%[101]

DCR: Disease control rate; DLT: dose-limiting toxicities; ICI: immune checkpoint inhibitor; MSI: microsatellite instability; ORR: objective response rate; OS: overall survival; PARPi: poly(ADP-ribose) polymerase inhibitors; PD-L1: programmed cell death ligand-1; PD-1: programmed cell death protein-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

higher number of TILs[51,52]. Other components of the TME include Tregs, MDSCs, tumour-associated macrophages (TAMs), and cytokines.

Tregs suppress effector T cells and APC via secretion of inhibitory cytokines, direct contact, and limiting inflammation[108]. The increased infiltration of Tregs into tumour cells has been observed in several other tumour types[109,110], and murine studies have demonstrated that depleting Tregs from the TME can help to restore antitumour immunity[109].

The presence of MDSCs in the TME has also been shown to promote angiogenesis, immune evasion, tumour growth and metastasis[108]. A study of patients with melanoma treated with CTLA-4 inhibitors

suggested that the increase in MDSCs was associated more often with non-responders[111]. Interestingly, the γ isoform of PI3K has been noted to be highly expressed in MDSC cells in a study of several cancer types, including breast cancer[112], and selectively inhibiting it can help to re-establish sensitivity to ICIs[113].

Another important group of cells present in the TME that promote immunosuppression and play a role in resistance to immunotherapy are TAMs, which consist of M1 and M2 macrophages[114]. M1 macrophages are mainly involved in antitumour immunity, while M2 macrophages are pro-tumourigenic. The accumulation of TAMs is regulated by cytokines, such as chemokine ligand 2 (CCL2), which was demonstrated by Qian *et al.* in their study using breast cancer- bearing murine model[115], as well as colony-stimulating factor-1 (CSF-1). It was observed to be correlated with increased macrophage infiltration and more frequent metastases in breast cancer patients[116]. Indeed, studies that evaluated CSF-1 receptor inhibition in combination with ICI treatment showed synergy of both agents and promising tumour regression, suggesting that CSF-1 receptor inhibitors can help to overcome tumour resistance to immunotherapy[117,118].

Besides individual populations of cells, the make-up of various cytokines present in the TME is also important in immune cell recruitment, activation, and proliferation by its balance of both stimulatory and suppressive effects[119]. For example, cytokines such as transforming growth factor β (TGF-β) induce immunosuppression by upregulating Tregs and inhibiting cytotoxic T lymphocytes[120]. Tumour cells also express ecto-5'-nucleotidase (CD73), which is an enzyme that dephosphorylates adenosine monophosphate (AMP), forming adenosine[121]. Adenosine is a potent immunosuppressor that binds to A2A receptors found on lymphocytes and suppresses its function[122]. Breast cancer cells have been shown to express CD73[123], and its expression appears to be regulated by the estrogen receptor (ER), whereby the loss of ER enhances the expression of CD73[124]. A proof of concept study confirmed that anti-CD73 antibody therapy can trigger adaptive antitumour immunity and inhibit metastasis in breast cancer[125].

Upregulation of other immune checkpoints

Resistance to ICIs can also be achieved via upregulation of other immune checkpoints such as T-cell immunoglobulin, mucin domain-3 protein (TIM-3), LAG-3, V-domain immunoglobulin suppressor of T-cell activation (VISTA), B and T lymphocyte attenuator (BTLA), and T-cell immunoreceptor tyrosine-based inhibition motif domain (TIGIT)[108,126-128]. The co-expression of multiple immune checkpoints has been demonstrated to be associated with T cell exhaustion, and subsequently resistance to ICIs[129]. Targeting these alternative pathways represents potential therapeutic options for overcoming drug resistance to ICIs. Although most studies evaluating such combination strategies have been in other tumour types such as melanoma and NSCLC[130-132], these are still relevant in breast cancers as epigenetic modifications resulting in upregulation of multiple immune checkpoints such as PD-L1, CTLA-4, TIM-3, and LAG-3 have been observed, and correlated with poorer patient prognosis in a study of breast cancer patients[133]. A study that specifically included breast cancer patients was a phase I study of LAG525, a monoclonal antibody blocking the binding of LAG-3 to MHC-II in combination with spartalizumab (an anti-PD-1 antibody) in patients with advanced malignancies, which showed durable responses[134]. In particular, 2 out of 5 patients with advanced TNBC showed a response, and in TNBC tumour biopsies, a trend in the conversion of immune-cold to immune-activated biomarker profiles was reported[134].

CONCLUSION: CHALLENGES AND FUTURE DIRECTIONS

Aside from resistance mechanisms to ICIs, there are also many unresolved and unanswered questions that have limited the use of ICIs in breast cancer. These include identifying the best predictive and prognostic biomarkers to guide treatment, evaluating the optimal duration of ICIs in the neoadjuvant/adjuvant setting,

and chemotherapy backbone in the metastatic setting, just to name a few. Recent review articles have discussed some of these topics[135,136].

Further advancement in this field needs to be led by sound science with good preclinical evidence from appropriate murine tumour models that can reflect the human immune environment. While this has conventionally largely been restricted due to a limited selection of murine tumour models, novel syngeneic tumour murine models have been better able to mirror the genomic heterogeneity of human cancer, and recapitulate the TME so as to provide accurate results. It is hoped that the use of appropriate novel syngeneic tumour murine models will allow us to further study ICI combinations effectively and accurately[137].

Lastly, studies looking beyond immunotherapy-based treatments are also being investigated. One such area is the study of the human gut microbiome, a host factor that influences not only the biology of tumour development but also the modulation of its response and resistance to immunotherapy[138-140]. Consequently, there are ongoing studies looking at modifying the gut microbiota in order to increase the efficacy of immunotherapy treatment. These include interventions such as the use of antibiotics, probiotics, faecal microbiota transplantation, and diet and prebiotics[141].

There is much to be anticipated in this evolving field of immunotherapy in breast cancer. While previously thought to be an immunologically "cold" cancer with limited responses to ICI, this is certainly set to change. The numerous ongoing trials evaluating ICIs in combination with novel therapies to overcome resistance and exploit the immune system, as well as the development of innovative immunomodulatory strategies, will allow us to further harness and expand the role of immunotherapy in breast cancer.

DECLARATIONS

Authors' contributions

Conceptualization: Wong RSJ, Ong RJM, Lim JSJ
Original draft writing: Wong RSJ, Ong RJM
Manuscript review and editing: Wong RSJ, Lim JSJ
All authors contributed to the article and approved the submitted version.

Availability of data and materials

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Conflicts of interest

JSJ Lim has received honoraria from Astra Zeneca, Novartis, Roche, DKSH, MSD, Eisai, Pierre Fabre; has advisory activity with Astra Zeneca, Novartis, Roche, DKSH, Pfizer and MSD; received research funding from CTI biopharma, Daiichi Sankyo and Synthon pharmaceuticals; and has received travel grants from Astra Zeneca and MSD.

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Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Emerging roles of 3D-culture systems in tackling tumor drug resistance

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Abstract

Drug resistance that affects patients universally is a major challenge in cancer therapy. The development of drug resistance in cancer cells is a multifactor event, and its process involves numerous mechanisms that allow these cells to evade the effect of treatments. As a result, the need to understand the molecular mechanisms underlying cancer drug sensitivity is imperative. Traditional 2D cell culture systems have been utilized to study drug resistance, but they often fail to mimic the 3D milieu and the architecture of real tissues and cell-cell interactions. As a result of this, 3D cell culture systems are now considered a comprehensive model to study drug resistance *in vitro*. Cancer cells exhibit an *in vivo* behavior when grown in a three-dimensional environment and react to therapy more physiologically. In this review, we discuss the relevance of main 3D culture systems in the study of potential approaches to overcome drug resistance and in the identification of personalized drug targets with the aim of developing patient-specific treatment strategies that can be put in place when resistance emerges.

Keywords: Drug resistance, 3D culture system, extracellular matrix, tumor microenvironment, personalized medicine

INTRODUCTION

One of the most significant challenges in cancer treatment is drug resistance. Cancerous cells can possess an intrinsic resistance to therapy or they can develop an acquired resistance to chemotherapy and other

targeted therapies, leading to treatment failure and disease progression[1-3]. The development of drug resistance in cancer cells is a multifactor event and involves numerous mechanisms that allow these cells to evade the effect of treatments. Intrinsic drug resistance can be defined as the innate resistance to drugs that are present inside the cells prior to the administration of any kind of treatment. It can be caused by (i) inherent genetic mutations that impair the response of cancer cells to drugs; (ii) intratumoral heterogeneity characterized by the presence of subclones, including cancer stem cells, which are insensitive to drugs; (iii) activation of defense mechanisms against environmental toxins. On the other hand, acquired resistance is defined as the progressive reduction of the efficacy of a treatment after its administration, which can be due to: (i) the surge of secondary mutations in proto-oncogenes that could become the new driver genes; (ii) mutation or dysregulation of the expression of the target of the therapy; (iii) dynamic alterations in the tumor microenvironment (TME). The mechanisms underlying intrinsic and acquired drug resistance could co-exist during tumor progression[3-5]. During the last decades, many different therapeutic approaches to overcome drug resistance have been tested. Combinatorial drug therapy (two or more drugs at the same time) has been used extensively and simultaneous multitargeting seems more effective in fighting drug resistance. It has been observed that cancers treated with the highest dosage of chemo- or targeted therapies rapidly become resistant to the treatment and new therapeutic approaches are based on "on and off" strategies. The intermittent treatment could interrupt the growth of resistant subpopulations inside the tumor[6]. Unfortunately, this kind of approach has also shown limitations and researchers are working hard to find new strategies to tackle drug resistance[3]. There is an urgent need to find new model systems to complement traditional 2D cell culture systems that are still the golden standard in the study of drug resistance. Researchers around the world are focusing on finding new systems to better model intrinsic and acquired resistance in tumors with the final aim of studying and tackling tumor drug resistance.

For years, cell culture systems have had an intense effect on the field of biomedical research for studying the molecular mechanisms of cancer progression and developing new therapies and treatments. Traditional 2D cell culture systems are the most commonly used preclinical models for different reasons: (i) easy to handle; (ii) relatively low costs; and (iii) suitable for high throughput analysis. Many anticancer drugs have been discovered thanks to the National Cancer Institute cancer cell line panel (NCI60), and the relevance of these models has been further supported by large pharmacogenomic screens such as the Genomics of Drug Sensitivity in Cancer, the Cancer Cell Line Encyclopedia, and the Cancer Therapeutics Response Portal[7-10]. Drug-adapted cancer cell lines are easily handled and they allow the establishment of a large number of models in a given timeframe and at a given cost. Even if the procedure is quite long (several months), the protocol is quite straightforward and the rate of success in establishing these models pretty high[11]. Clinically relevant mechanisms of resistance have been discovered using these models[12]; however, they cannot mimic intra-tumor heterogeneity, the 3D milieu, and the architecture of real tissues and cell-cell interactions. As a result, 3D cell culture systems are nowadays considered a more comprehensive model to study drug resistance *in vitro*. For example, the heterogeneous traits of a tumor, such as hypoxia, genetic status, and altered gene expression, can be more genuinely analyzed in 3D models rather than in 2D models[13,14]. Consequently, using 3D cell culture systems can find more reliable and accurate outcomes. However, the setting of protocols for 3D models is quite a time-consuming procedure and not successful for all kinds of tumors.

ADVANTAGES OF 3D CELL CULTURE SYSTEMS

3D cell culture systems have recently emerged as a better option than conventional 2D cell culture systems for disease modeling, drug screening, and cancer research. In the section below, we want to briefly discuss the advantages of 3D cell culture over 2D cell culture.

3D cell culture systems mimic the *in vivo* microenvironment in comparison to 2D cell culture systems since they contain a higher degree of cellular organization, cell-cell interactions, and extracellular matrix (ECM) components. 3D cell cultures show a well-defined geometry, which could be directly related to function. Furthermore, inside these cultures, proliferating, non-proliferating, and necrotic cells are present, as in intact human tumors. In 3D cell systems, multicellular and multi-layered systems can be created and exploited to study the interactions between different cell types[15-17]. A wide variety of organoids, involving mini-brains, intestinal tissue, and liver tissue, can be generated and organoids made of various cell types can mimic the organization of an organ, making them ideal for drug screening and drug response[18]. The cellular heterogeneity obtained in 3D cultures models mass transport limitations typical of solid tumors. This allows for the designing of more precise models of disease, which can help develop effective treatments[19]. On the same line, 3D cell culture systems offer the potential for personalized medicine, enabling the production of patient-specific organoids for personalized drug screening and treatment[20].

3D cell culture systems can potentially offer better predictive outcomes for patients' drug responses[21]. They are more suitable for evaluating drug bioactivity since they simulate the impacts of treatments compared to 2D cell cultures more precisely[22]. Additionally, they are a more biologically applicable system for toxicology screening, as 3D systems provide more tissue-like structures and better simulate patients' status, which can help predict the effects of toxins more accurately[23]. They are ideal, for example, for studying the influences of nanoparticles on cells, providing a more realistic test environment for nanotoxicity studies[24].

Cell shape and environment are recognized as crucial in determining cell behavior and gene expression of a cell. In epithelial tumors, for example, polarity is essential, together with the formation of tight junctions and desmosomes linked to cell proliferation and tumorigenesis. In a 3D context, cells acquire a "normal-like" architecture and gene expression profile, while 2D monolayers on artificial support fail to maintain the original epithelial cell characteristics[25]. Cancer stem cells, which are crucial for treatment response, are strongly dependent on the niche for differentiation. Inducing stem cell differentiation and tissue-specific functionality is a challenging process in 2D cell culture systems, while 3D cell culture systems can be used for this purpose[26,27]. An important feature of 3D cell cultures is the remarkable control over the growth and differentiation of cells in complex tissue or organ-like structures that could be achieved, aiding researchers in simulating multifaceted disorder organization or physiological environments[21].

3D cell culture systems are better tools for studying cell migration and invasion. Cells in 2D cell culture systems often move in a flat 2D environment, while cells in 3D cultures can exhibit more natural 3D migration and invasion, allowing for more precise modeling of cancer metastasis[28]. Moreover, 3D cell culture systems can be used to study the impacts of mechanical forces on cancer cell movement[29]. In theory, 3D cell culture systems can also be used to create substantial quantities of functional tissue, which may be used for transplantation or tissue engineering. This is specifically useful for applications such as skin grafts and bone regeneration[30].

Even though 2D systems have been extensively used to study drug resistance due to their easy handling and relatively low costs, in the last few years, 3D culture systems have attracted the interest of researchers. Acquired resistance relies on different mechanisms, such as secondary mutations of proto-oncogenes or mutations or dysregulation of the expression of the target of the therapy and alterations in the TME[3]. 3D systems could help in the study of this phenomenon. For example, RNA editing levels are significantly correlated with drug sensitivity in cancer cell lines and can be heavily influenced by tumor environment[31]. By studying RNA editing in 3D cell culture models, researchers are able to investigate the relationship between RNA editing and drug sensitivity/resistance in a more physiologically relevant context.

Table 1 briefly summarizes the main differences between 2D and 3D culture systems.

DIFFERENT TYPES OF 3D CELL CULTURE SYSTEMS

The most relevant components of the tumor stroma are ECM proteins and stroma cells. The ability of cancer cells to proliferate, migrate, adhere, differentiate and the activation of specific cell signaling pathways strongly rely on changes in ECM composition[32] and on the interactions of tumor cells with stroma cells[33]. Cancer-associated fibroblasts (CAFs) are the most representative component in the tumor stroma, and they enhance cancer cell survival and the ability of cell invasion. They are involved in ECM remodeling and tumor metabolic rewiring, and contribute to the onset of drug resistance[34]. Mesenchymal stem cells (MSCs), which support the epithelial mesenchymal transition (EMT) of various cancer cells, alter the immunocompetence of the TME, inducing drug resistance in tumor cells. Various immune cells such as tumor-associated macrophages (TAMs), lymphocytes, natural killer (NK) cells, and dendritic cells (DCs) have pivotal roles in tumor control. Crosstalk between endothelial cells (ECs) and tumor cells during the formation of new blood vessels is crucial in providing the required nutrients and oxygen for the growth of tumors[33] [Figure 1]. Considering all these important interactions within the TME, cell-based 3D models have emerged as models which could closely recapitulate physiological tumor organization *in vitro*.

Essential cell-ECM interactions can be recapitulated by biomimetic scaffolds, where tumor cells are seeded inside a 3D platform made of a porous biomaterial where they attach, start to grow, rearrange, and secrete ECM. After this process, called "scaffold maturation", the entire scaffold is completely covered by cells [Figure 2A]. Biomaterials present in scaffolds can be synthetic polymers, i.e., polyethylene glycol (PEG), polycaprolactone (PCL), poly(hydroxyethylmethacrylate) (PHEMA), poly(lactic-co-glycolic acid) (PLGA), and ceramics (i.e., hydroxyapatite or bioglass), which are often preferred over natural polymers because their properties can be more easily controlled[35] and they can be functionalized by the addition of peptides which can modulate protein adsorption as well as cell adhesion[36]. As far as natural biomaterials are concerned, collagen, fibrin, alginate, and chitosan can be derived from tissue and cells[35]. Decellularized native tissues potentially allow for an easier recapitulation of tumor tissue and ECM architecture being closer to the *in vivo* condition; however, the decellularization process can be challenging since various steps (i.e., detergent and enzyme digestion) may affect tissue architecture.

Hydrogels are crosslinked networks formed by hydrophilic polymers connected through physical, ionic, or covalent interactions that highly resemble ECM[37] and allow cells to behave and communicate in an *in vivo* setting[35]. At first, cells are mixed with a precursor hydrogel, and then the hydrogel is crosslinked in order to obtain a cell-laden hydrogel. Cells start to grow and rearrange in the mature hydrogel, where they form cell clusters and secrete ECM, while the original hydrogel architecture remains intact [Figure 2B].

Scaffolds that are used to create complex 3D models can be obtained by 3D bioprinting, with the advantages of well-defined architecture, composition, and high reproducibility[38]. A cell-laden bioink made of a precursor hydrogel and cells is prepared and deposited in a preprogrammed pattern using a 3D bioprinter; hydrogel is then crosslinked to form the final structure [Figure 2C]. Printing may be obtained through extrusion, inkjet, and stereolithography, as well as laser-assisted and electrospinning-based bioprinting[39]. Scalability is one of the interesting features of 3D printed models. Indeed, it is possible to take into consideration 4D variables. Time, for example, is crucial for assessing the kinetics of growth factors, drugs or for following tumor cell dissemination over time[40]. Prominent issues in the use of 3D printing techniques could be damaging pressure and excessive heating during the printing of living cells, as well as slow printing speeds and the continuous need for new biocompatible and printable bioinks[41]. At the moment, however, insufficient reproducibility to create reference models is the main limitation. Despite

Table 1. Comparison of the main features of 2D and 3D culture systems

Aspect	2D cell culture	3D cell culture
Cell arrangement	Cells grow in a monolayer on a flat surface	Organoids, hydrogels, and other three-dimensional structures, such as scaffolds, allow cells to grow in a more natural spatial environment.
Cell-cell interactions	Limited	Improved, allowing for direct interaction between cells, cell signaling, and sophisticated cellular actions.
Cell-matrix interactions	Limited	An ECM that mimics the *in vivo* microenvironment interacts with cells, managing cell adhesion, migration, and differentiation.
Phenotypic expression	Changed compared to *in vivo* conditions	Provides a more accurate representation of *in vivo* environments, including tissue-specific gene expression, protein synthesis, and cellular responses.
Cellular functions	Basic, absent of tissue-specific functions and complex cell-cell interactions	It is possible to accomplish more intricate and organotypic processes, such as cell polarization, differentiation, barrier function, and the creation of tissue-specific substances.
Spatial organization	Homogeneous distribution of cells	Cells are capable of spatial organization, resulting in multicellular structures, gradients, and tissue-like structures.
Drug response	Useful to study different drug sensitivities in comparison to *in vivo* conditions	Improved cellular responses and interactions across various cell types provide better predictive capability for drug screening, enabling assessment of medication efficacy and toxicity.
Disease modeling	Limited capability to recapitulate intricate diseases and tissue interactions	Enables the creation of disease models that are more accurate, making it easier to research disease progression, find new drugs, and practice personalized treatment.
High-throughput screening	Well-suited for high-throughput analyses and screening purposes	Due to 3D culture's intricacy and additional experimental requirements, it is typically less suitable for high-throughput screening.

ECM: Extracellular matrix.

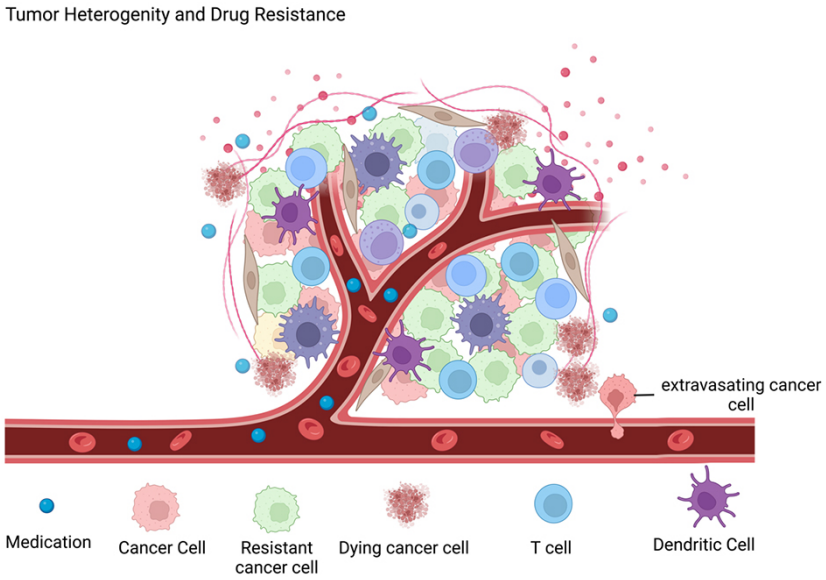

Figure 1. Tumor heterogeneity and drug resistance. Primary tumors are heterogeneous. Subpopulations of cancer cells showing partial or full resistance to therapy are present. The activation of resistance mechanisms can be due to the activation of genetic or epigenetic mechanisms that could be caused by the therapy itself and by the interaction of tumor cells with the microenvironment. This figure is generated with Biorender.com.

these drawbacks, 3D bioprinting is a powerful technique since cancer and stroma cells are embedded in a complex microenvironment where tumor-stroma cell interactions, tumor-ECM contacts, and self-organization of the tissue could be deeply studied.

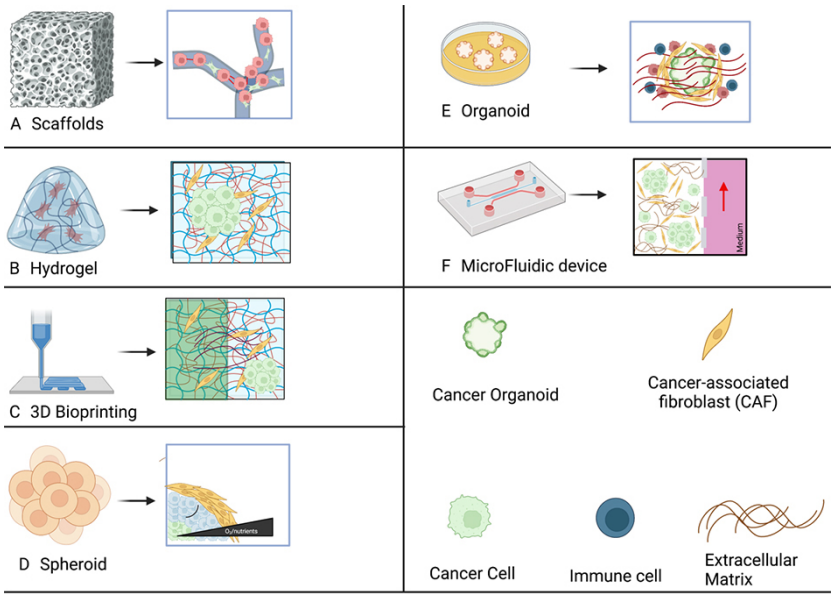

Figure 2. Schematic drawing of different 3D culture systems describing the main features of each system. (A) Scaffold. Cells, including immune cells, adhere to the scaffold, where they can proliferate and produce ECM, which coats the entire scaffold; (B) Hydrogel. Cells are combined with a hydrogel solution, which produces a sturdy framework that can support the cells. Cells divide and reorganize themselves; (C) 3D bioprinting. Cells are combined with hydrogel to produce bioink, a substance that can be printed using a 3D bioprinter. Depending on the type of bioink used, multiple cultures, including scaffold-based, scaffold-free, and semi-scaffold-free cultures, can be obtained; (D) Spheroid. Cells with significant cell-cell interaction are allowed to aggregate. As cells multiply, they rearrange themselves, and dense spheroids with oxygen or nutrition gradients could develop. Different kinds of cells can be combined in the spheroid; (E) Organoid. Cells are cultured in a hydrogel environment utilizing materials like Matrigel. Organoids are produced and are surrounded by other cells that replicate and produce natural ECM. Immune cell penetration can be simulated by adding immune cells to the culture; (F) Microfluidic device. Cells, spheroid, or organoids are plated onto the ready platform together with hydrogel, and then a medium containing nutrients can flow and perfuse the cells. Different kinds of cells can be added to the culture. This figure is generated with Biorender.com. ECM: Extracellular matrix.

Spheroids [Figure 2D] contain important features that characterize tumors, such as cellular heterogeneity, gene expression variations, cell signaling pathway alterations, cell-cell, cell/ECM interactions, and multicellular layer organization, thus mimicking *in vivo* tumor morphology[42]. For these reasons, they are widely used to study tumor biology and to evaluate anticancer drugs. Spheroids can be generated easily by hanging drop methods or spontaneous cell aggregation of cells grown on low-attachment surfaces; however, more sophisticated techniques, such as 3D bioprinting or magnetic levitation, are applied to obtain more homogenous spheroid populations both in size and number[43]. Bigger spheroids (500 μm in diameter) can be used to recapitulate the milieu where micro-metastases develop since nutrients and oxygen are limited in those big structures[44]. Spheroids are useful models to study cancers that form tumor embolus or cancers characterized by packed tumor cell clusters such as inflammatory breast cancers[45,46]. In recent years, spheroid models have been implemented by the combination of different cell types in the same spheroids, i.e., tumor cells, monocytes, and CAFs, taking advantage of a particular technique utilizing spinner flasks[47]. In order to evaluate the role of pancreatic stellate cells (PSCs) in pancreatic ductal adenocarcinoma (PDAC) cells resistance to gemcitabine and c-MET inhibitors, 3D-spheroid co-cultures of primary human PDAC cells and PSC (heterospheroids) were generated and compared with spheroids containing only PDAC cells (homospheroids). While heterospheroids were more resistant to gemcitabine compared to homospheroids, no difference was observed for c-MET inhibitor treatment, suggesting that the choice of 2D or 3D systems to study drug resistance is strongly dependent on the kind of drug under investigation[48]. The main limitations of the use of spheroids are the poor uniformity in size/morphology of the obtained spheroids and often the difficulty in retrieving the cells for further molecular analysis[46].

Spheroids are formed by forcing cell aggregation[49], whereas organoids are obtained from progenitor cells that can proliferate, differentiate, and self-organize, thus closely recapitulating the 3D structure of the *in vivo* tissue/tumor from which they originate. As a result of this natural process, organoids preserve cancer cell heterogeneity as well as genetic and phenotypic properties of the tumor of origin [Figure 2E]. The significant feature of organoids is that they can be obtained from the patient's cells and represent a potential alternative to patient-derived tumor xenografts (PDX) and animal models in many aspects, i.e., they are less laborious and expensive[50]. However, it has been observed that organoids could be helpful in predicting the responsiveness to therapy for some kinds of drugs but not for all. Ooft *et al.* developed patient-derived organoids (PDOs) from metastatic lesions of colorectal cancer (CRC) to identify non-responders to CRC standard therapy. While PDOs were able to predict the response in more than 80% of patients treated with irinotecan-based therapies, they failed to predict the response to 5-fluorouracil plus oxaliplatin, underlying once again the relevance of the choice of the system to study drug resistance[51]. To implement organoid models, co-culture of PSCs and PDAC tumor organoids has been established, thus allowing the differentiation of PSCs into myofibroblast-like and inflammatory CAFs[52]. More sophisticated organoid cultures can be obtained by 3D bioprinting[53]. The main drawback of organoids is that, in some kinds of tumors, they are unable to reach *in vivo*-like levels, and often, the variability can be high between experiments. Lastly, organoids lack vasculature and stroma[18].

Both cell-ECM and cell-based 3D models described so far have an important limitation: cells cannot be perfused inside the 3D organization. Microfluidics devices have been introduced in tumor modeling to overcome this issue. They consist of a network of channels that allows the control and modification of various parameters, for instance, mechanical forces, cell localization, and chemical gradients [Figure 2F]. Spheroids or organoids can be grown inside these platforms and thanks to the control of several factors, microfluidics can model tumor tissues and organs so these systems are called organ-on-a-chip devices. Another important point is that a small volume of reagents can be used due to miniaturization, thus reducing the costs of these kinds of cultures compared to the other 3D-culture methods[54]. High-throughput screenings can be performed with increased controllability in microfluidics. The advent of 3D bioprinting has speeded up the process and reduced the costs. However, there is an urgent need for new materials to produce the chips, since the most common material used for this, polydimethylsiloxane (PMDS), can absorb small molecules in a nonspecific way. Microfluidics can be used to study tumor-stroma cell interactions because different cell types can be cultured in a chip[54]. The possibility to tightly control multiple gradient candidates makes microfluidics the model of choice to analyze the effects of growth factors or drugs in a biomimetic microenvironment. The main limitation of microfluidics is that the fabrication of these devices requires specialized skills.

Table 2 highlights the main characteristics of the different 3D systems described above.

APPLICATIONS OF 3D CULTURE SYSTEMS IN DRUG RESISTANCE RESEARCH

3D cell culture systems have drawn substantial attention in the study of various aspects of drug resistance. Drug response and the mechanisms of drug resistance can be assessed using 3D cell culture models in a setting that is more physiologically suitable. Altered cell signaling, genetic variations, and tumor microenvironment interactions contribute to lower drug sensitivity due to tumor heterogeneity[55,56].

3D cell culture platforms can be used for the screening and identification of new therapeutic agents that can overcome drug resistance. Through the use of these models, compound libraries may be tested in a more accurate tumor microenvironment, which helps researchers find new treatment combinations that are more effective at eliminating cancer cells that show intrinsic or acquired resistance. Ultimately, organoids and

Table 2. Comparison among the different 3D-culture techniques

Technique	Description	Advantages	Disadvantages	Ref.
Scaffolds	Cells are grown on or inside a three-dimensional scaffold structure	Mimics the structure of tissue and encourages cell differentiation	Limited control over the characteristics of the scaffold	[37]
3D bioprinting	A bioprinter is used to deposit cells layer by layer to produce intricate three-dimensional structures	Precise cell placement control; customizable	High-priced; limited scalability	[40]
Spheroids	Without an external scaffold, cells self-assemble into 3D spherical structures	Easy and inexpensive to produce; repeatable	Limited scalability, limited nutrient, and oxygen diffusion	[54]
Organoids	Three-dimensional self-organizing cell formations that resemble the functions and structures of organs	Reflects the complexity of the organ and models disease	Variability, complexity, and time-consuming generation	[18]
Microfluidics	Microscale channels that simulate *in vivo* tissue settings are used to cultivate cells, giving researchers great control over the culture environment.	High-throughput screening and accurate gradient control	Required technical knowledge; restricted scalability	[53]

tumor explants are examples of patient-derived 3D cell culture models that can be used to assess how each patient responds to a particular treatment. With the use of this strategy, precision oncology techniques can be guided by the discovery of individualized treatment plans and the prediction of patient-specific drug responses[55].

3D cell culture systems to overcome drug resistance

Intratumor heterogeneity poses a significant obstacle to effective cancer therapy. Tumor subpopulations may respond differently to therapeutic interventions due to intrinsic drug resistance or the emergence of acquired drug resistance. Therefore, innovative research models to comprehend and address this complexity are needed.

CRC exhibits distinct subpopulations within clonal organoids: cells generating large spheroids (D-pattern) and cells generating small spheroids (L-pattern). S-pattern spheroids display chemotherapy resistance, but modulation of Notch signaling can push them towards the D-pattern, offering a potential therapeutic target to reverse chemoresistance[57]. The nuclear tyrosine-protein kinase receptor 3 (TYRO3) receptor tyrosine kinase has been identified as an inducer of drug resistance and metastasis in CRC organoid culture and mouse models. TYRO3 function requires matrix metalloproteinase-2 (MMP-2) and bromodomain-containing protein 3 (BRD3), making selective inhibition of MMP-2 or BRD3 activity a potential strategy to ameliorate CRC malignancy[58]. In understanding drug resistance mechanisms, mutational status plays a crucial role. For instance, KRAS codon G12 (KRASG12) mutations in metastatic CRC (mCRC) patients have been associated with increased resistance to trifluridine/tipiracil chemotherapy. This observation was paralleled in isogenic cell lines and PDOs, highlighting the relevance of 3D systems in studying drug resistance[59]. In microsatellite instability-high (MSI-H) CRC, inflammation plays a pivotal role in disease progression and immunosuppression. *In silico* investigation highlighted a correlation between inflammatory conditions and poor response to programmed cell death-1 (PD-1) blockade. Cultures of paired T cells and organoid cells from patients confirmed this hypothesis, and single-cell RNA sequencing revealed the involvement of neutrophils in the suppressive immune microenvironment. An elevated neutrophil/lymphocyte ratio was associated with an impaired immune status and a poor response to immunotherapy, suggesting it could potentially serve as an indicator for clinical decision-making[60]. In MSI-H CRC, resistance to immune checkpoint blockade has been observed in a specific subtype characterized by peritoneal metastases and ascites formation. To study the mechanism of immune checkpoint blockade, PDOs were transplanted into the cecum of humanized mice. It was found that immune checkpoint blockade led to reduced tumor masses and liver metastasis, driven by the formation of tertiary lymphoid structures (TLS) containing B cells, T cells, and an activated interferon-γ signaling pathway. However, peritoneal metastases lacked B cells and TLS, and T cells displayed a dysfunctional phenotype[61].

3D cell culture systems, such as organotypic tumor spheroids and matched PDOs, have been instrumental in identifying effective treatment strategies to overcome resistance to cancer immunotherapy in other cancers. For instance, Sun *et al.* identified TANK-binding kinase 1 (TBK1) as a potent therapeutic target to enhance the response to PD-1 blockade in melanoma and other cancers. Inhibition of TBK1 reduced the cytotoxicity threshold to effector cytokines, thereby empowering the response to PD-1 blockade[62].

Resistance to common chemotherapy treatments, such as 5-fluorouracil and cisplatin (5FU + CDDP), remains a major challenge. As mentioned above, RNA editing is correlated with the emergence of resistance[31]; organoid lines from resistant patients with the intestinal subtype of gastric cancer (GC) showed upregulation of JAK/Src-signal transducer and activator of the transcription (STAT) signaling and adenosine deaminases acting on RNA 1 (ADAR1), along with hyper-edited lipid metabolism genes due to A-to-I editing on the 3'UTR of stearoyl- CoA desaturase (SCD1). SCD1 favored lipid droplet formation, reducing chemotherapy-induced ER stress and enhancing self-renewal in resistant GC lines[63]. In another subset of GC known as stem-like/Epithelial-to-mesenchymal transition/Mesenchymal (SEM-type) GC, resistance to glutaminolysis inhibition was observed due to a stem-like population in the tumor. SEM tumors displayed high glutaminase (GLS) levels and upregulation of the 3 phosphoglycerate dehydrogenase (PHGDH)-mediated mitochondrial folate cycle pathway to produce NADPH. A potential treatment strategy to combat chemotherapy resistance in SEM-type GC involves the combined inhibition of GLS and PHGDH to eliminate stemness-high cancer cells[64].

Chemoresistance in PDAC is quite common, and there is an urgent need to identify new targets and compounds to improve treatment outcomes. A biobank of human PDAC organoid models was established and used to screen FDA-approved compounds, leading to the discovery of irbesartan, an angiotensin type 1 (AT1) receptor antagonist. Irbesartan inhibits the Hippo/YAP1 pathway, reducing c-Jun expression and enhancing chemotherapy effectiveness in killing PDAC cells. High c-Jun expression in PDAC patients was associated with poor response to the standard chemotherapy regimen (gemcitabine plus nab-paclitaxel)[65]. Loss of cyclin dependent kinase inhibitor 2A (CDKN2A) (encoding p16INK4A) and activation of KRAS play crucial roles in PDAC development and malignant growth. Restoration of p16INK4A with CDK4/6 inhibitors (CDK4/6i) alone has shown limited efficacy in clinics. However, combinatorial treatment with a CDK4/i and an ERK-MAPK inhibitor synergistically suppresses tumor growth through blocking CDK4/6i-induced compensatory upregulation of ERK, PI3K, antiapoptotic signaling, and MYC expression in PDAC cell lines and organoids[66]. Neoadjuvant chemotherapy (neoCTx) is used to treat PDAC, but its effects vary among patients. PDOs generated from PDAC tissues allowed researchers to evaluate differential responses to FOLFIRINOX or Gem/Pac regimens. This approach could help personalize poly-chemotherapy regimens, avoiding severe side effects and increasing the number of patients who benefit from complete neoadjuvant treatment[67].

TME strongly influences the treatment outcome and new therapies targeting the cells of the TME are emerging. PDAC and cholangiocarcinoma (CCA) progression and chemoresistance are influenced by CAFs. In co-cultures of primary PDAC organoids and patient-matched CAFs, CAFs displayed a pro-inflammatory phenotype, while organoids showed increased expression of genes associated with EMT and drug resistance. This suggests that targeting CAFs could improve treatment sensitivity in PDAC and CCA[68]. CAFs also contribute to drug resistance in CCA. In PDOs consisting of epithelial and matched CAFs, CAFs were relatively resistant to bortezomib treatment due to an overexpression of CXCR4. However, the addition of a CXCR4 inhibitor reversed the resistance to bortezomib in CAFs and sensitized CCA to anti-PD1 treatment, offering a promising triple treatment strategy for CCA patients[69].

Pyroptosis is a gasdermin-driven lytic programmed cell death triggered by inflammatory caspases that can be put to good use to kill cancer cells, including those exhibiting chemo- or targeted therapy resistance. Su *et al.* have demonstrated that pyroptosis can be reactivated in resistant pancreatic and lung cancer cell lines and organoids by administering a Src or ceramidase inhibitor. In resistant cancer cells, the β5-integrin protein plays a crucial role in controlling chemotherapy-induced pyroptosis, leading to chemoresistance. This effect is mediated through the upregulation of the sphingolipid metabolic enzyme ceramidase (ASAH2) expression, which is regulated by the STAT3 signaling pathway. The increased ceramidase expression results in a reduction of the metabolite ceramide concentration and subsequent suppression of reactive oxygen species (ROS) production, effectively blocking chemotherapy-induced canonical pyroptosis[70].

In conclusion, intratumor heterogeneity presents a significant obstacle to the development of effective cancer therapies. There are now more ways to understand and deal with this complexity thanks to the use of sophisticated models such as organoids and 3D cultures. These models have highlighted possible treatment targets and drug combinations to potentially overcome drug resistance.

3D cell culture systems to investigate drug resistance mechanisms

Inhibitors of receptor tyrosine kinases (RTKIs) are commonly used in cancer treatment. However, in head and neck squamous cell carcinoma (HNSCC), despite the high expression of epidermal growth factor receptor (EGFR), RTKI treatment often fails to show therapeutic efficacy in clinical trials, questioning their inclusion in standard therapy regimens. To understand the reasons behind these failures, researchers evaluated the response of HNSCC cell lines to RTK inhibitors under both 2D and 3D cell culture conditions. Interestingly, the HNSCC cells displayed strong resistance to lapatinib, an RTKI, when cultured in 3D conditions. However, in a 2D setting, the same cells responded to the lapatinib treatment. This resistance was associated with an overexpression of HER3. These results indicate that the increased cell-to-cell contacts and enhanced communication between cells due to higher cell density, as well as the augmented concentration of receptors and intracellular signaling molecules of the EGFR family in 3D systems, could impact drug response. Moreover, in 3D systems, cells live in hypoxic and nutrient-poor conditions and behave as dormant cells that are less susceptible to cytostatic treatment, leading to increased resistance. This finding indicates that the culture conditions can alter cell signaling pathways, potentially leading to different drug resistance mechanisms in cancer therapy[71].

Glioblastoma (GBM) is a challenging cancer with poor patient prognosis and frequent tumor recurrence. Due to the resistance of certain subpopulations, such as mesenchymal and glioma stem cells, to the standard chemotherapy drug temozolomide (TMZ). Small protein kinase inhibitors have also been extensively studied for GBM treatment, but their benefit for patients has been limited compared to standard therapy regimens. Fabro *et al.* investigated the effects of prolonged treatment with TMZ, enzastaurin, and imatinib on patient-derived GBM 2D and 3D organotypic multicellular spheroid models. They observed a heterogeneous inter-patient response to the different drugs, with minor changes in kinase activation, primarily associated with the ErbB signaling pathway. Additionally, they identified a new resistance mechanism to imatinib treatment in one 3D sample, resulting in a more invasive behavior. The authors suggest the stroma cell interactions present in 3D structures could exert a protective effect on tumor cells against TMZ action[72]. In other studies, using 3D collagen scaffold culture and 3D Ca-alginate scaffolds, researchers analyzed the gene expression profiles of GBM cells. Glioma cells cultured in 3D collagen scaffolds exhibited increased colony and sphere formation and increased drug resistance compared with 2D cultures. The hub genes involved in 3D collagen-induced drug resistance (AKT1, ATM, CASP3, CCND1, EGFR, PARP1, and TP53) were predicted by bioinformatics and their expression was verified by Western Blot analysis[73].

Glioma cells cultured in 3D Ca-alginate scaffolds exhibited significant changes in gene expression compared with 2D cultures, with upregulation of genes related to mitogen-activated protein kinase signaling, autophagy, drug metabolism through cytochrome P450, and ATP-binding cassette transporter, and downregulation of genes related to the cell cycle and DNA replication. This altered gene expression could be due to the fact that the 3D-collagen culture may enhance the stemness traits of glioma cells compared to 2D conditions[74]. These findings provide valuable insights into the differences in gene expression between 2D and 3D culture systems and their potential implications for drug resistance in GBM.

Biomimetic collagen scaffolds have been used as models to study the tumor hypoxic state and may be valuable tools in predicting chemotherapy responses in breast cancer (BC). Triple negative (TN) and luminal A BC cells were treated with doxorubicin in 2D cultures, 3D collagen scaffolds, or orthotopically transplanted murine models. In 3D culture conditions, TN cells displayed impaired drug uptake, increased drug efflux, and drug lysosomal confinement, contributing to drug resistance. Luminal A cells, on the other hand, were found to be insensitive to DNA damage due to deregulation of the p53 pathway. Transcriptome analysis identified a signature of deregulated genes that were validated in BC patients, showing their potential as predictive biomarkers. Transporter associated with antigen processing 1 (TAP1) and tumor protein p53-inducible protein 3 (TP53I3) showed high expression and were associated with shorter relapse in ER+ breast tumor patients, while high expression of lysosomal-associated membrane protein 1 (LAMP1) was associated with the same clinical outcome in TNBC patients. Resveratrol treatment with subsequent hypoxia inhibition partially re-sensitized cells to doxorubicin treatment, highlighting the relevance of these 3D preclinical models in the study of resistance mechanisms. Conversely, data obtained in monolayers were not able to recapitulate *in vivo* conditions and efficacy was overestimated when tested in a 2D context[75]. To investigate recurrence mechanisms in ER+ BC, tumor organoids were generated from needle biopsy, surgical excision, and cerebrospinal fluid samples. Next generation sequencing (NGS) analysis revealed detrimental mutations in PIK3CA and TP53 genes and mutations of unknown functions in BAP1, RET, AXIN2, and PPP2R2A. Drug screening in BC organoids allowed for the evaluation of drug toxicity and showed dynamic changes in tumor progression, reflecting the heterogeneity of BC and demonstrating their reliability as models for personalized medicine development[76]. In ER+ positive BC cells, the recurrent deletion of 16q12.2 affects AKTIP, which governs tumorigenesis, specifically in ER+ positive BC cells. Its deletion is linked to ERα protein level and activity and triggers JAK2-STAT3 activation, an alternative survival signal in the absence of ERα activation. Consequently, ERα-positive PDOs become more resistant to ERα antagonists, but this resistance can be overcome by co-inhibition of the JAK2/STAT3 signaling pathway[77].

These papers show the applications of 3D cell culture platforms in the study of the mechanisms behind treatment resistance in various cancers in comparison with 2D systems. In order to design targeted therapies, it is now possible to identify specific molecular pathways and cellular interactions that contribute to resistance through the use of 3D models.

Potential use of 3D cell culture systems to identify individual drug response

In recent years, personalized medicine has made considerable progress, allowing tailored treatments for each patient. One of the potential advancements in personalized medicine is the development of 3D cell culture models, which could hold the potential for addressing individual drug resistance.

Anderle *et al.* pioneered the creation of a 3D system of epithelial ovarian cancer (EOC) comprising patient-derived microtumors and autologous tumor-infiltrating lymphocytes (TILs). By employing reverse-phase protein array (RPPA) analysis of over 110 total and phospho-proteins on these models, they measured

patient-specific sensitivities to standard platinum-based therapy and predicted individual treatment responses. The inclusion of autologous TILs in 3D cultures facilitated testing patients' responses to immune checkpoint inhibitors (ICIs). The therapeutic sensitivity predictions obtained through 3D systems hold clinical relevance post-surgery for patients' treatment. Ongoing follow-up studies in larger cohorts aim to validate the effectiveness of this platform for guiding clinical decision-making[78].

A significant milestone was achieved by Senkowski *et al.*, who leveraged viably biobanked tissues to establish organoids from high-grade serous ovarian cancer (HGSC). These organoids faithfully recapitulated the original tumors in terms of both genetics and phenotype, as evidenced by genomic, histologic, and single-cell transcriptomic analyses. Furthermore, when cultured in a human plasma-like medium, organoid drug responses correlated with clinical treatment outcomes, highlighting the potential of these models for predicting patient responses to therapy[79].

The significance of 3D systems in predicting drug responses and the development of resistance has led to the establishment of international consortia conducting multicenter studies to validate the clinical relevance of these models.

The INFORM program, an international precision oncology initiative, enrolled 132 pediatric cancer patients with relapsed or refractory conditions. In a two-year pilot study, fresh tumor tissue spheroid cultures were exposed to a library of clinically relevant drugs. The drug sensitivity profile (DSP) results from the multicellular tumor tissue spheroid cultures correlated with known molecular targets (BRAF, ALK, MET, and TP53 status). Remarkably, drug vulnerabilities were identified in 80% of cases, and the correlation between clinical outcomes and DSP results in selected patients suggests the potential advantage of this platform in predicting individual treatment responses[80].

3D models are also proving useful in predicting individual responses to radiotherapy (RT) in various tumor types. For instance, Lee *et al.* cultured HNSCC patient tumor cells in a 3D pillar/well array culture system, exposing them to standard radiation protocols and evaluating their RT response. This approach allowed the quantification of the radioresponse index in HNSCC patients[81]. Similarly, a HNSCC organoid biobank comprising 110 models, including 65 tumor models, was established. Organoids exposed to chemo-radiotherapy and targeted therapies demonstrated drug response correlations with patient clinical outcomes. Notably, *in vitro*, organoid response to RT closely mirrored patients' clinical responses[82].

In the context of mCRC, which often develops resistance and has limited therapeutic options, tumor-derived organoids are being used to assess individual drug sensitivity and explore new treatment avenues. In a phase 2 study involving 90 mCRC patients with progression after standard therapy, organoids derived from metastatic biopsies were cultured and evaluated for sensitivity to a panel of drugs. Patients were treated with the drug demonstrating the highest relative activity, resulting in a 50% progression-free rate at two months[83]. Another study generated organoids from 40 mCRC patients and performed drug screenings, associating the results with patients' responses. In the future, these findings may support the potential use of organoids to generate functional data and to aid in clinical decision-making[84]. The integration of PDO drug response with multi-omics data could likely lead to the identification of proteomic and gene expression signatures capable of predicting treatment response or resistance in advanced CRC. Drug sensitivity tests coupled with mass spectrometry and RNA-seq analysis revealed differential responses to oxaliplatin and palbociclib. Oxaliplatin resistance was linked to t-RNA aminoacylation processes, while high palbociclib responses were associated with MYC activation and T-complex protein ring complex (TriC) chaperonin protein enrichment[85].

The complexity of protocols to generate PDOs has posed a challenge, prompting efforts to standardize these procedures. A novel microfluidics-based system known as the Pu·MA System has been introduced, coupled with high-content imaging and metabolite analysis, for the processing and multi-functional profiling of tumoroid samples from metaplastic BC subtype patients. High-content imaging and multi-parametric profiling revealed tumoroid sensitivity to specific drugs, closely mirroring primary tumor responses[86]. In inflammatory BC, a living organoid biobank was established from locally advanced patients undergoing neoadjuvant chemotherapy. Organoids treated with neoadjuvant drugs demonstrated a response pattern that closely matched patients' clinical responses, suggesting that PDOs could predict neoadjuvant therapy outcomes in BC patients[87].

Predicting clinical treatment responses in locally advanced or metastatic lung cancer (LC) patients using tumor organoids has also been explored. Wang *et al.* generated 212 LC organoids and conducted drug sensitivity tests for chemotherapy and targeted therapy. Organoids successfully predicted clinical treatment responses[88]. In another study by Mazzocchi *et al.*, 3D LC organoids were fabricated from pleural effusion aspirate, a rare cell source. These organoids recapitulated lung tissue anatomy and exhibited lung-specific behavior compared to 2D cultures. While 2D cultures were more sensitive to chemotherapy, organoids better reflected the *in vivo* situation, underlining the relevance of 3D systems in studying drug responses and resistance emergence[89].

In the context of GBM, the need for assays that predict drug responses prompted the establishment of a high-density 3D primary cell culture model from resected GBM tissue. These cultures accurately modeled GBM heterogeneity, including tumor and surrounding cells, and replicated histopathological traits of parent tumors. These 3D cultures effectively predicted chemotherapy responses within a brief period and correlated with patients' responses to TMZ therapy[90]. Metabolic imaging based on NAD(P)H fluorescence lifetime imaging microscopy (FLIM) was applied to GBM organoids to predict anticancer treatment responses. This technique identified TMZ Responder and Non-Responder tumors shortly after surgery, with metabolic stratification aligning with molecular levels, demonstrating its potential for patient stratification[91].

PDOs have also proven valuable in predicting responses to neoadjuvant chemotherapy (NAT) in PDAC patients. A PDO biobank was generated from patients receiving NAT and untreated patients. The response to NAT correlated with PDO chemotherapy response (oxaliplatin), highlighting the feasibility of rapid PDO drug screening shortly after tissue resection for optimal patient NAT regimen selection[92].

Through standardization and integration with multi-omics data, 3D cell culture models offer improved patient outcomes across various cancer types, enabling precise prediction of drug reactions, therapeutic outcomes, and personalized treatment options.

CONCLUSION

Overall, 3D cell culture models may be of help in understanding the general and the patients' specific mechanisms of drug resistance by providing more physiologically relevant systems for disease modeling and drug screening. These models could allow for the identification of personalized drug targets and the potential development of patient-specific treatment strategies. However, standardization of culture protocols, characterization methods, and outcome metrics is essential for maximizing the clinical value of 3D cell culture models. For example, the choice of bio-materials used to generate 3D cultures is critical for the successful generation of organoids and the prediction of drug response. The comparison among patient-derived BC cells encapsulated in bioprinted PEG-derived hydrogels and gelatin-derived hydrogels (GelMA

and GelSH) showed that GelSH increased organoid formation ability and a better response to doxorubicin, EP31670, and paclitaxel treatments compared to 2D cultures and other matrices[36]. To ensure repeatability among laboratories, efforts are being made to create standards and quality control procedures[93]. Integrating 3D cell culture models with multi-omics data (genomics, transcriptomics, and proteomics) can provide a thorough understanding of disease causes and treatment responses. The combination of these datasets can enable the identification of molecular signatures and biomarkers for patient stratification and therapy choice[94]. However, the fact that the 3D models cannot be used for all drugs without distinction needs to be considered. It has been shown, for instance, that organoids fail to predict the response to 5-fluorouracil plus oxaliplatin, while they are able to predict the response in more than 80% of patients treated with irinotecan-based therapies[51]. Therefore, for some kinds of drugs, 2D culture models remain the elective systems to study the mechanisms of drug resistance[12]. Despite this, 3D cell culture models hold tremendous potential for improving therapeutic approaches and ultimately enhancing patients' outcomes.

DECLARATIONS

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Authors' contributions

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Conflicts of interest

All authors declared that there are no conflicts of interest.

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Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Supramolecular host-guest nanosystems for overcoming cancer drug resistance

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Abstract

Cancer drug resistance has become one of the main challenges for the failure of chemotherapy, greatly limiting the selection and use of anticancer drugs and dashing the hopes of cancer patients. The emergence of supramolecular host-guest nanosystems has brought the field of supramolecular chemistry into the nanoworld, providing a potential solution to this challenge. Compared with conventional chemotherapeutic platforms, supramolecular host-guest nanosystems can reverse cancer drug resistance by increasing drug uptake, reducing drug efflux, activating drugs, and inhibiting DNA repair. Herein, we summarize the research progress of supramolecular host-guest nanosystems for overcoming cancer drug resistance and discuss the future research direction in this field. It is hoped that this review will provide more positive references for overcoming cancer drug resistance and promoting the development of supramolecular host-guest nanosystems.

Keywords: Supramolecular nanosystems, host-guest interaction, cancer drug resistance

INTRODUCTION

With the number of cancer cases increasing each year, cancer has become the second leading cause of death worldwide[1]. Although chemotherapy remains the primary method of cancer treatment, its effectiveness is severely limited by cancer drug resistance[2-5]. The occurrence of cancer drug resistance is associated with

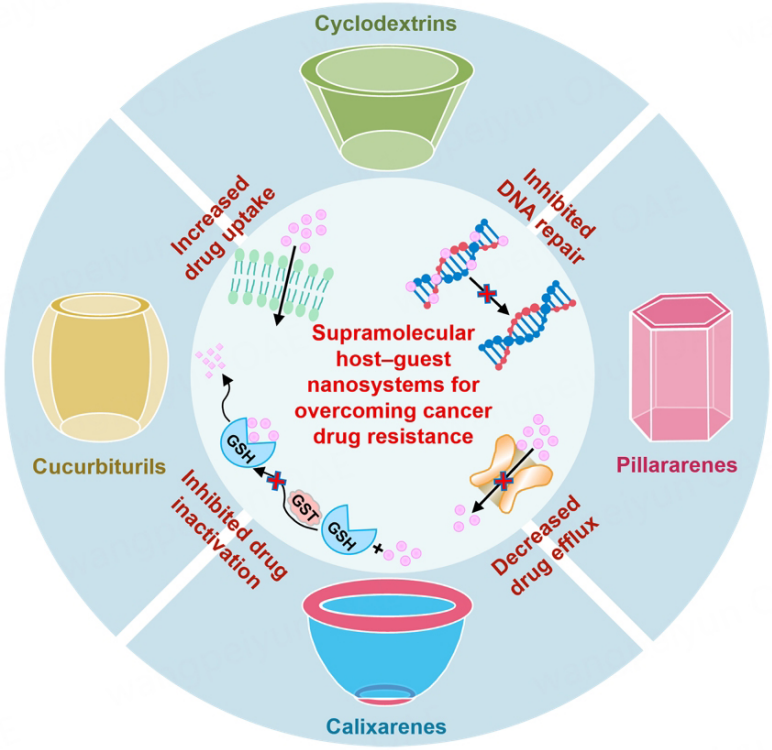
multiple factors, including the overexpression of multidrug resistance gene (MDR1), anti-apoptotic protein (BCL-2), multidrug resistance-associated protein (MRP), and the enhanced activity of glutathione S-transferase (GST) and DNA repair enzyme[6-8]. These factors can lead to decreased drug uptake, increased drug efflux, DNA damage repair, abnormal drug metabolism, and dysfunctional apoptosis, resulting in cancer drug resistance[9,10]. Nanosystems have been widely used to overcome cancer drug resistance due to their ability to alter the way drugs enter cells, increase drug uptake, and improve drug stability[11,12]. Common nanosystems used to overcome cancer drug resistance include liposomes, polymeric nanoparticles, and metal nanoparticles. However, there are still some problems in the application of these nanosystems. For example, drugs loaded in liposomes tend to leak in the circulatory system before reaching the tumor; polymeric nanoparticles have a high burst release effect; and metal nanoparticles have poor biocompatibility. These problems have led to the limited role of these nanosystems in overcoming cancer drug resistance[13,14]. Therefore, it is urgent to develop a class of novel nanosystems to reverse cancer drug resistance.

Supramolecular chemistry is "chemistry beyond the molecule"[15]. Supramolecules generally refer to organized aggregates formed by non-covalent interactions of two or more molecules, including electrostatic interaction, hydrogen bond, van der Waals force, and π-π interaction[16-21]. By introducing supramolecules into the nanosystem, it is possible to construct a more promising new drug delivery system, supramolecular host-guest nanosystem, which provides a potential solution for cancer drug resistance[22-28]. Compared with traditional nanomaterials constructed by covalent interactions, supramolecular host-guest nanomaterials constructed by non-covalent interactions have excellent dynamic reversibility and responsiveness to various stimuli (such as weak acidity, specific enzymes, and different redox environments)[29-33]. Based on these advantages, supramolecular host-guest nanosystems can increase drug uptake, accurately release drugs, inhibit drug efflux, and protect the activity of drugs, which provide great possibilities for eliminating cancer drug resistance and promoting the progress of cancer treatment[34-37].

In this review, we summarize the research progress of supramolecular host-guest nanosystems for overcoming cancer drug resistance over the past few years, including cyclodextrins, calixarenes, cucurbiturils, and pillararenes [Scheme 1]. Moreover, the challenges and prospects of supramolecular host-guest nanosystems for overcoming cancer drug resistance are discussed extensively. This review aims to provide valuable insights and contribute to the development of more effective ways to reverse cancer drug resistance.

CYCLODEXTRINS-BASED HOST-GUEST NANOSYSTEMS FOR OVERCOMING CANCER DRUG RESISTANCE

Cyclodextrins (CDs), a class of natural oligosaccharides obtained from the degradation of starch, are linked by glucopyranose units through α-1,4-glycosidic bonds [Figure 1][38,39]. The most common CDs contain six, seven, and eight glucopyranose units, respectively, known as α, β, and γ-CDs[40,41]. CDs have hydrophobic cavities, which can encapsulate hydrophobic drug molecules to form host-guest complexes[42-45]. In addition, these complexes can self-assemble into nanoparticles, greatly improving the efficiency of the drug (such as good water solubility, high stability, and low physiological toxicity)[46-48]. Therefore, CDs-based host-guest nanosystems have the potential to reverse cancer drug resistance by increasing drug uptake and decreasing drug efflux[49-51]. For example, Yang *et al.* constructed three nanomedicines based on β-CDs that enhanced the drug uptake and the toxicity of drug-resistant cells[52]. Das *et al.* prepared a dual-responsive nanocarrier by embedding carbon nanotubes into β-CDs-based polymers, enabling the combination of cocktail chemotherapy with photothermal therapy, which was conducive to multidrug resistance reversal[53].



Scheme 1. Supramolecular host-guest nanosystems for overcoming cancer drug resistance. GSH: Glutathione; GST: glutathione S-transferase.

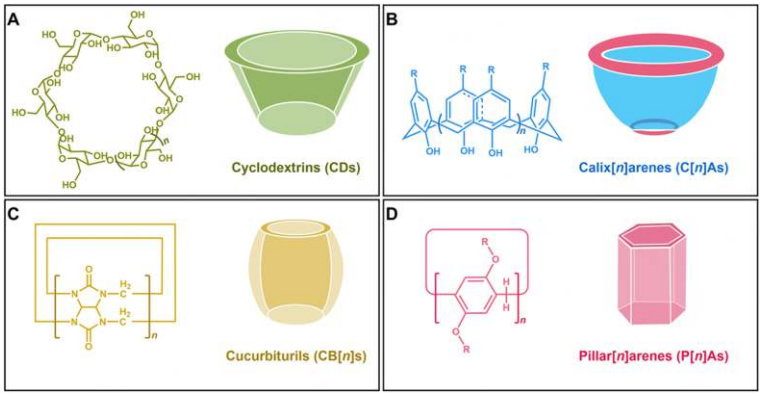


Figure 1. Schematic illustration of structures of (A) CDs; (B) calixarenes (C[*n*]As); (C) cucurbiturils (CB[*n*]s); and (D) pillararenes (P[*n*]As).

P-glycoprotein (P-gp) is an energy-dependent efflux pump located on the cell membrane[54,55]. P-gp depends on the energy produced by ATP hydrolysis within the mitochondria to keep intracellular drug concentrations low by transporting drug molecules outside the cell, resulting in drug resistance[56-58]. Therefore, drug resistance can be effectively reversed by inducing mitochondrial dysfunction. Wang *et al.* constructed a nanosystem (Aa-DOX + ADD@PC) based on a pH-sensitive graft copolymer (PBAE-g-β-CD) to achieve co-loading of the anticancer drug doxorubicin (DOX) and mitochondrial inhibitor (ADD) [Figure 2A][59]. When Aa-DOX + ADD@PC was endocytosed by tumor cells, DOX and ADD were released in the acidic environment for combined chemotherapy. Western blot assay was used to study the expression

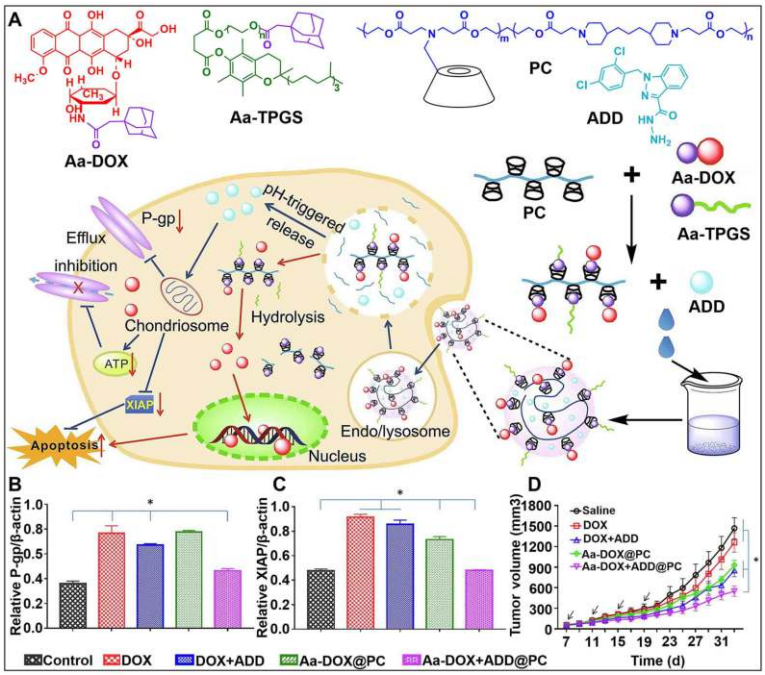


Figure 2. (A) Schematic illustration of dual-drug co-loaded nanoparticle (Aa-DOX + ADD@PC) for overcoming cancer drug resistance; The expression levels of (B) P-gp and (C) XIAP in MCF-7/ADR cells with different treatments; (D) Tumor growth inhibition curves of tumor-bearing mice after various formulations. (*$P < 0.05$) This figure is quoted with permission from Wang *et al.*[59]. ADD: Mitochondrial inhibitor; DOX: doxorubicin; XIAP: X-linked inhibitor of apoptosis protein.

levels of P-gp and X-linked inhibitor of apoptosis protein (XIAP), and it was found that Aa-DOX + ADD@PC showed the best inhibitory effect on P-gp and XIAP [Figures 2B and C]. Moreover, the therapeutic effect of Aa-DOX + ADD@PC was better than that of free DOX, significantly inhibiting the growth of drug-resistant tumors [Figure 2D]. In this work, the effective loading of mitochondrial inhibitors by CDs was used to successfully reverse drug resistance by decreasing drug efflux, providing a new therapeutic platform for overcoming multidrug resistance (MDR).

Furthermore, compared with free drugs, tumor cells can effectively take up nanomedicine, which is conducive to the reversal of drug resistance caused by low intracellular drug concentration[60,61]. Liu *et al.* developed pH/redox dual-responsive DOX delivery nanosystems (DOX@RPMSNs) based on cationic β-cyclodextrin-PEI (PEI-β-CD) to overcome drug resistance of tumor cells [Figure 3A][62]. The poly (ethylene glycol) amine derivative shell (PEG-b-PLLDA) of DOX@RPMSNs could protect DOX@RPMSNs from safely reaching the vicinity of tumor cells, increasing the absorption of drugs by tumor cells. PEI-β-CD and DOX were sequentially released in response to the action of acid and glutathione (GSH) in tumor cells. DOX was used to kill tumor cells, and PEI-β-CD acted as an inhibitor to downregulate the expression of drug resistance-related P-gp by reducing ATP [Figure 3B]. Compared with other formulations, DOX@RPMSNs significantly inhibited tumor growth [Figures 3C and D]. These results indicated that DOX@RPMSNs successfully improved drug resistance reversal.

Histone-acetyltransferase (GCN5) is a silencing protein closely related to drug-resistance genes. Drug resistance caused by efflux can be reversed by down-regulating the expression of GCN5[63,64]. RNA interference (RNAi) is a therapeutic technique that specifically targets mRNA and regulates the expression of silencing proteins[65-67]. Yuan *et al.* exploited a nanosystem (DOX/siRNA@HPMSNs) to combine RNAi and DOX, which could knockout drug-resistance genes (Figure 3E)[68]. The hyaluronan (HA) shell of DOX/

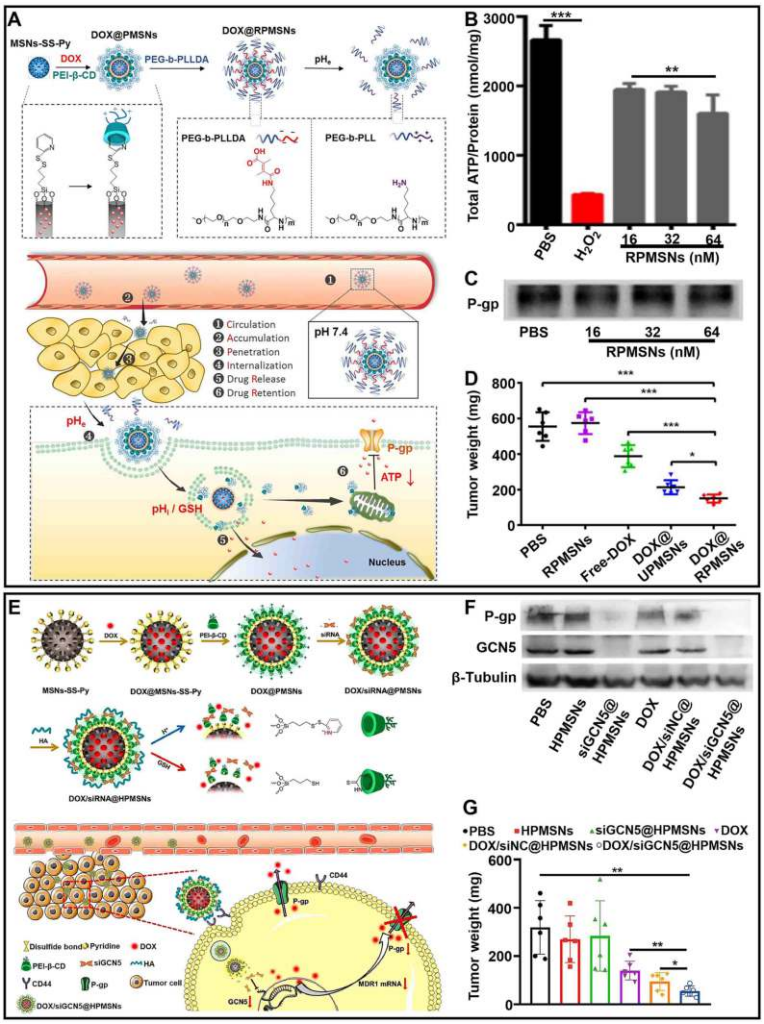


Figure 3. (A) Schematic illustration of the construction of sequentially responsive nanosystem (DOX@RPMSNs) and dual-responsive drug release; (B) Total ATP concentrations of MCF7/ADR cells treated with H_2O_2 and different doses of RPMSNs; (C) The expression levels of P-gp in tumor cells with different treatments; (D) Changes of tumor volume in tumor-bearing mice with different treatments ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). This figure is quoted with permission from Liu *et al.*[62]; (E) Schematic illustration of the construction of co-delivery nanosystem (HPMSNs) and dual-responsive drug release; (F) The expression levels of P-gp and GCN5 in tumor cells with different treatments; (G) Changes of tumor weight in tumor-bearing mice with different treatments ($^{*}P < 0.05$, $^{**}P < 0.01$). This figure is quoted with permission from Yuan *et al.*[68]. DOX: Doxorubicin; PEG-b-PLLDA: poly (ethylene glycol) amine derivative shell.

siRNA@HPMSNs could prolong the circulation time of DOX/siRNA@HPMSNs *in vivo* and target tumor cells, which promoted the accumulation of antitumor drugs. In the microenvironment of tumor cells, the effective release of siRNA could downregulate the expression of GCN5 to reduce the efflux of DOX caused by P-gp [Figure 3F]. Additionally, the inhibition rate of DOX/siRNA@HPMSNs on the growth of drug-resistance tumors was higher than DOX by evaluating the chemotherapeutic effects of different drug delivery systems [Figure 3G]. The two pH/redox dual-responsive nanosystems reduced drug efflux caused by the overexpression of P-gp in different ways, providing more possibilities for reversing MDR.

The acidic tumor microenvironment commonly found in solid tumors can reduce the endocytosis of free drugs and dissociate drug molecules[69,70]. Therefore, pH-responsive supramolecular host-guest nanosystems have been widely developed to enhance cell internalization and protect drugs from dissociation[71-73]. He *et al.* prepared a pH-responsive nanoparticle (Ac-α-CD NP) based on acetylated α-CD (Ac-α-CD), which

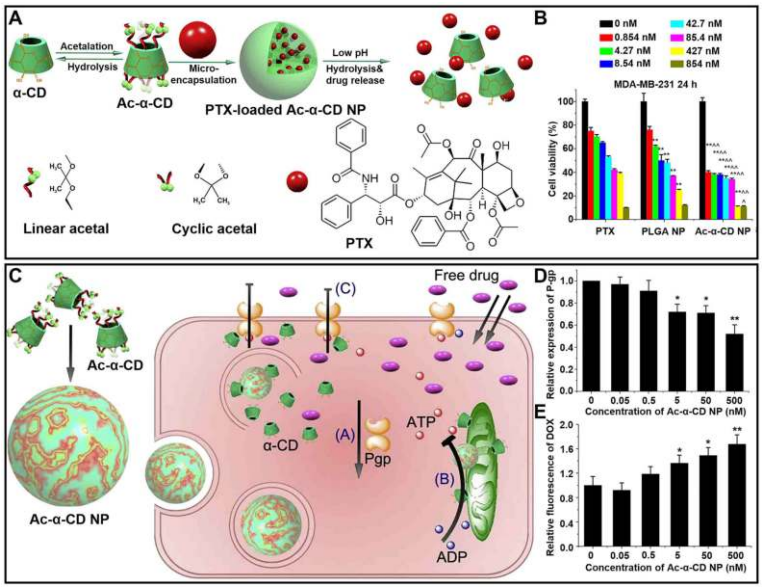


Figure 4. (A) Schematic illustration of the formation of pH-sensitive nanosystems (Ac-α-CD NP); (B) The cell viability of MDA-MB-231 cells treated with different doses of PTX, PLGA NP, and Ac-α-CD NP ($^{**}P < 0.01$). This figure is quoted with permission from He *et al.*[74]; (C) Schematic illustration of the antitumor progress of Ac-α-CD NP in MCF-7/ADR cells; (D) The P-gp expression level; and (E) the accumulation of DOX with different doses of Ac-α-CD NP in MCF-7/ADR cells ($^{*}P < 0.05$, $^{**}P < 0.01$). This figure is quoted with permission from Shi *et al.*[75]. CD: Cyclodextrin; DOX: doxorubicin; NP: nanoparticle; PTX: paclitaxel.

could stably encapsulate paclitaxel (PTX) [Figure 4A][74]. Ac-α-CD NP had a stronger inhibitory effect on the viability of breast cancer drug-resistant cells (MDA-MB-231) compared with free PTX and PLGA NPs [Figure 4B]. Moreover, Ac-α-CD NP exhibited good drug activity at a low concentration (0.854 nM), indicating its potential to kill drug-resistant cells.

Additionally, further studies showed that Ac-α-CD NP could also enhance the uptake and sensitivity of drug-resistant cells to DOX [Figure 4C][75]. α-CD was released by pH-induced hydrolysis of Ac-α-CD NP to inhibit the expression of P-gp and decrease the activity of ATPase, eliminating drug resistance caused by drug efflux [Figure 4D]. The changes in drug concentrations indicated that the downregulation of P-gp expression directly increased the accumulation of DOX in drug-resistant cells, thus achieving the purpose of inhibiting cancer drug resistance [Figure 4E]. Such pH-responsive supramolecular nanoparticles could not only inhibit the viability of drug-resistant cells at low concentrations but also increase the uptake and sensitization of drug-resistant cells to DOX, successfully reversing drug resistance from multiple angles.

Various star-shaped polymers can be obtained by modifying β-CD with different polymer chains[76,77]. These polymers can further self-assemble into stable supramolecular host-guest nanoparticles after loading anticancer drugs[78-80]. Compared with free drugs, nanoparticles are more easily internalized by tumor cells, reducing the efflux of drugs[81-83]. These factors work together to eliminate cancer drug resistance[84,85]. Chen *et al.* constructed a cationic β-CD-based nanocarrier that co-delivered PTX and Nur77 gene (an orphan nuclear receptor) to eliminate cancer drug resistance[86]. In addition, they reported a nanoparticle based on a PEGylated star-shaped copolymer successfully reversed MDR1-induced drug resistance[87].

Subsequently, they designed a new type of thermosensitive star-shaped polymer β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$ with "V"-shaped arms, which encapsulated PTX through the cavity of β-CD [Figure 5A][88]. The drug-loaded polymer further self-assembled into a stable supramolecular host-guest nanomedicine at

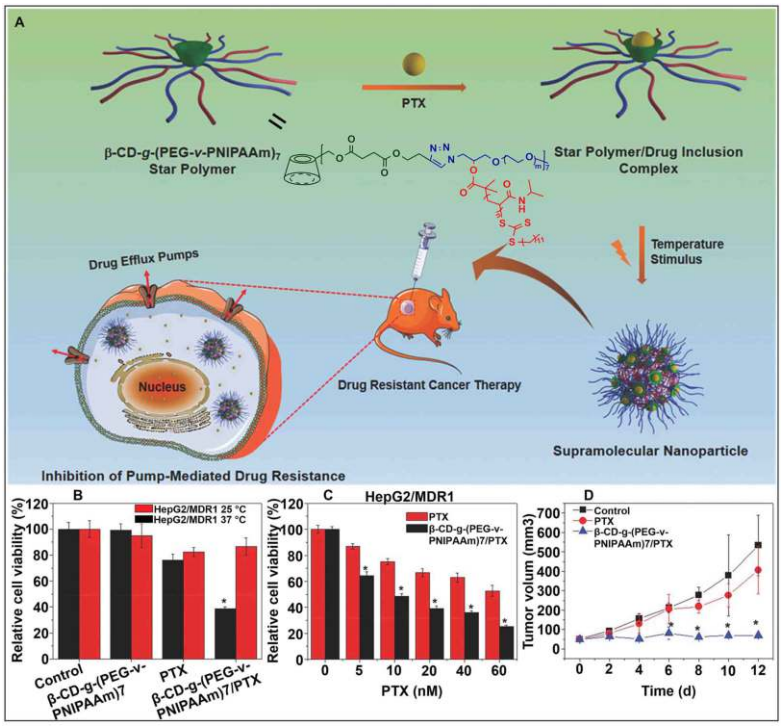


Figure 5. (A) Schematic illustration of supramolecular nanoparticle for inhibiting pump-mediated drug resistance; (B) Cell viability of HepG2/MDR1 cells treated with β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$, PTX, and β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$/PTX at different temperatures; (C) Changes in cell viability of HepG2/MDR1 cells treated with different doses of PTX and β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$/PTX; (D) Tumor growth inhibition curves of tumor-bearing mice after various treatments ($^*P < 0.05$). This figure is quoted with permission from Fan *et al.*[88]. CD: Cyclodextrin; MDR1: multidrug resistance gene; PEG: poly (ethylene glycol); PTX: paclitaxel.

37 °C, greatly enhancing the retention of drugs in cells. Compared with other drugs, this supramolecular host-guest nanomedicine was more sensitive to the change of temperature, causing a sharp decline in cell viability at 37 °C [Figure 5B]. When the drug-resistance tumor was transplanted into mice and treated with PTX and nanomedicine, respectively, β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$/PTX was more prominent in reducing cell viability [Figure 5C]. Additionally, there was no obvious change in tumor volume after treatment with β-CD-*g*-(PEG-*v*-PNIPAAm)$_7$/PTX [Figure 5D]. These results indicated that β-CD-based temperature-responsive nanomedicine had a good therapeutic efficacy against drug-resistant tumors.

Moreover, Li *et al.* constructed a unimolecular micelle based on a star-shaped polymer (β-CD-*g*-PCL-SS-PEG-FA) that stably encapsulated DOX [Figure 6A][89]. The folic acid (FA) in the unimolecular micelle could target and penetrate tumor cells to increase the accumulation of DOX, inhibiting the cancer drug resistance caused by decreased drug uptake. The drug loaded in the unimolecular micelle could be released in response to GSH. MTT assay analysis indicated that β-CD-*g*-PCL-SS-PEG-FA had a better inhibitory effect on cell viability compared with free DOX [Figure 6B and C]. In addition, the overexpression of folate receptors on cervical cancer drug-resistant cells (HeLa/MDR1) accelerated the uptake of β-CD-*g*-PCL-SS-PEG-FA, enhancing the therapeutic effect of DOX on drug-resistant cells. Such β-CD-based stimuli-responsive supramolecular host-guest nanoparticles showed exciting results in overcoming cancer drug resistance due to the precise targeting, effective uptake, and controlled release of drugs.

CALIXARENES-BASED HOST-GUEST NANOSYSTEMS FOR OVERCOMING CANCER DRUG RESISTANCE

Calixarenes are a class of cyclic oligomers formed by methylene-bridging ortho-phenolic hydroxyl groups

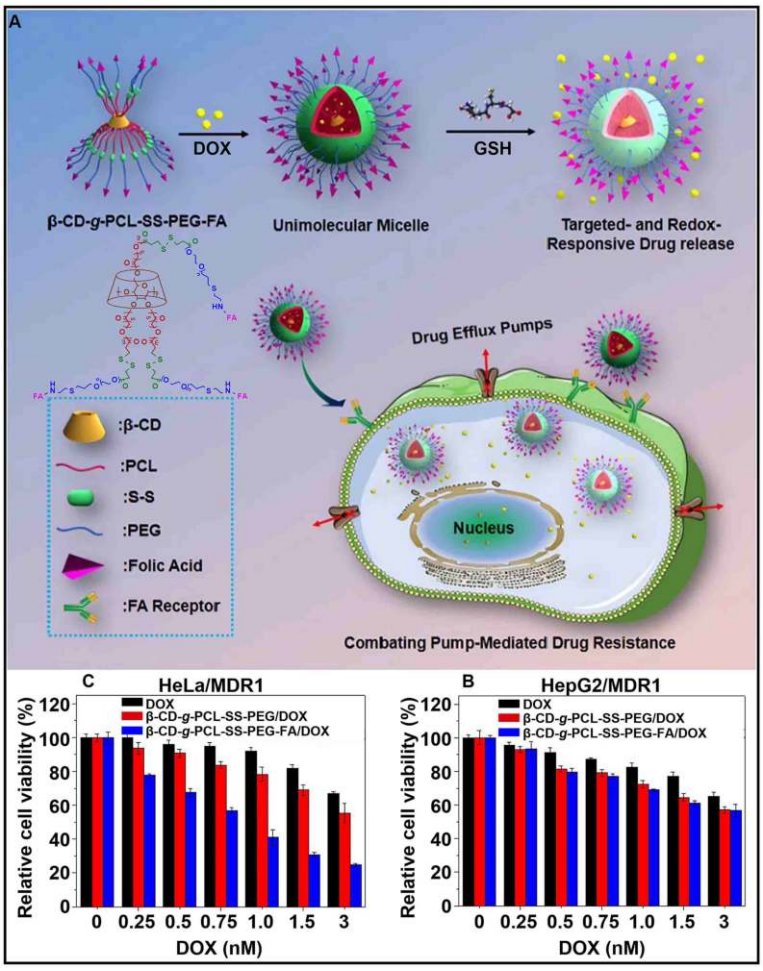


Figure 6. (A) Schematic illustration of a unimolecular micelle for inhibiting pump-mediated drug resistance; Cell viability of (B) HepG2/MDR1 cells and (C) HeLa/MDR1 cells treated with different doses of DOX, β-CD-*g*-PLC-SS-PEG/DOX, and β-CD-*g*-PLC-SS-PEG-FA/DOX. This figure is quoted with permission from Li *et al.*[89]. CD: Cyclodextrin; DOX: doxorubicin; FA: folic acid; GSH: glutathione; MDR1: multidrug resistance gene; PEG: poly (ethylene glycol).

[Figure 1B][90-92]. Due to their molecular shapes similar to the Sangreal, they are named calix[*n*]arenes (C[*n*]As) by Gutsche[93]. By introducing hydrophilic and hydrophobic groups at the upper and lower rims of C[*n*]As, respectively, amphiphilic C[*n*]As can be designed, which are easy to self-assemble into vesicles, nanoparticles, or other aggregates[94-99]. Host-guest nanosystems based on C[*n*]As have low toxicity and good biocompatibility, becoming a new research hotspot in the field of cancer drug resistance[100-102].

The close coordination between GSH and GST can initiate detoxification mechanisms within tumor cells, leading to the formation of drug resistance[103-105]. For example, GST can catalyze the binding of GSH to electrophilic antitumor drugs, accelerating the degradation of drugs[106-108]. Therefore, drug resistance can be reversed by regulating the GST. Recently, Dai *et al.* designed a nanomedicine (Pt-cCAV$_{5\text{-FU}}$) based on sulfonatocalix[4]arene for overcoming GST-induced cancer drug resistance [Figure 7A][109]. The GST regulator (5-FU) was encased into the hydrophilic core of Pt-cCAV$_{5\text{-FU}}$ self-assembled from a host-guest complex of sulfonatocalix[4]arene with cisplatin. Pt-cCAV$_{5\text{-FU}}$ actively released cisplatin and 5-FU during the hydrolysis process caused by esterase. 5-FU could downregulate GST activity, prompting cisplatin to damage DNA rather than binding to GSH [Figure 7B]. In addition, the endocytosis of cisplatin resistance cells A549/CDDP against Pt-cCAV$_{5\text{-FU}}$ was stronger than that of A549, which increased the accumulation of

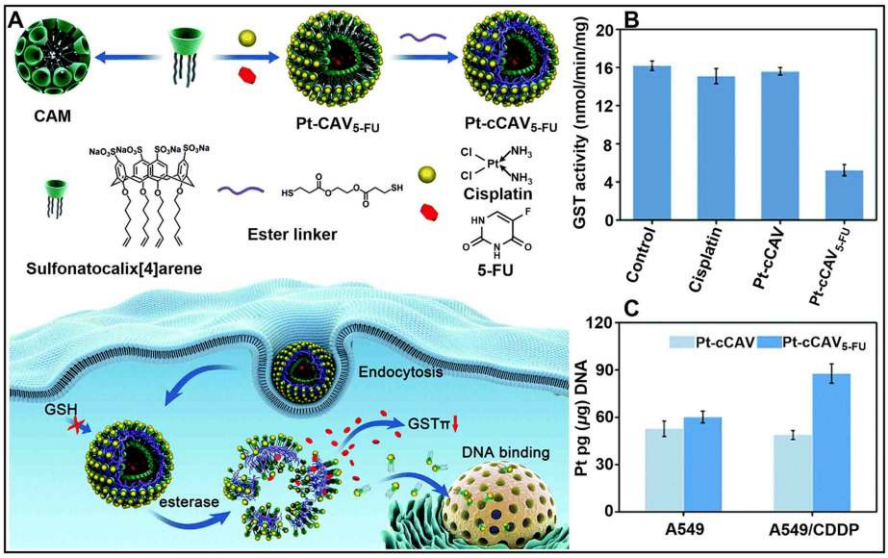


Figure 7. (A) Schematic illustration of Pt-cCAV$_{5\text{-FU}}$ for overcoming cisplatin resistance in A549/CDDP cells; (B) The GST activity of A549/CDDP cells with different treatments; (C) Platinum content in the genomic DNA of A549 and A549/CDDP cells after incubation with Pt-cCAV and Pt-cCAV$_{5\text{-FU}}$ for 12 h. This figure is quoted with permission from Dai *et al.*[109]. GSH: Glutathione; GST: glutathione S-transferase.

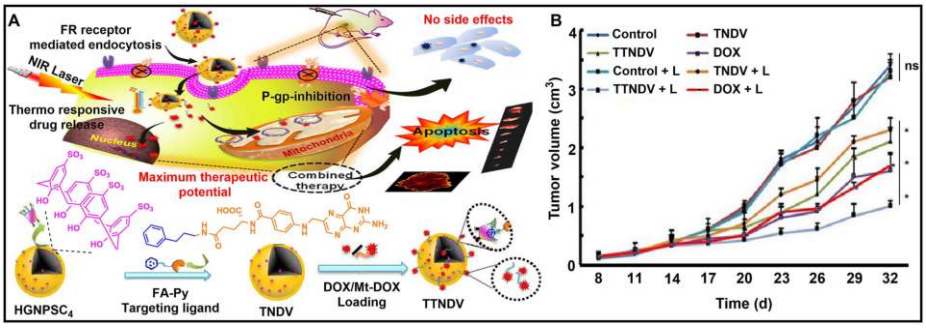


Figure 8. (A) Schematic illustration of TTNDV to overcome cancer drug resistance by killing n-DNA and Mt-DNA; (B) Tumor growth inhibition curves of tumor-bearing mice after different formulations ($^*P < 0.05$). This figure is quoted with permission from Nair *et al.*[114]. DOX: Doxorubicin; FA: folic acid.

cisplatin in cancer cells [Figure 7C]. All of these factors ultimately made Pt-cCAV$_{5\text{-FU}}$ more toxic to drug-resistance cells. This work developed a novel nanomedicine, laying the foundation for C[*n*]As-based host-guest nanosystems to reverse cisplatin resistance.

Furthermore, although free DOX can kill the nuclear DNA (n-DNA) of tumor cells, the undamaged mitochondrial DNA (Mt-DNA) can trigger drug resistance[110-112]. Therefore, the design of a drug to destroy synchronously n-DNA and Mt-DNA can promote the reversal of drug resistance[113]. Nair *et al.* constructed a gold nanotherapy platform (TTNDV) based on sulfonatocalix[4]arene [Figure 8A][114]. The nanoplatform could encapsulate DOX and mitochondrion-targeted analogue (Mt-DOX) in an optimal ratio of 1:100 to reverse cancer drug resistance caused by mitochondrial escape. *In vitro* and *in vivo* experiments showed that TTNDV had less toxic side effects than free DOX. In addition, TTNDV had a stimulating response to temperature. Under near-infrared irradiation, drugs embedded in TTNDV were released simultaneously to kill n-DNA and Mt-DNA, successfully overcoming DOX resistance and improving the chemotherapeutic effect of DOX [Figure 8B]. This work solved the problem of DOX resistance by killing Mt-DNA to induce

apoptosis, providing a feasible strategy for reversing cancer drug resistance.

CUCURBITURILS-BASED HOST-GUEST NANOSYSTEMS FOR OVERCOMING CANCER DRUG RESISTANCE

Cucurbiturils (CB[*n*]s) are a kind of macrocyclic hosts constructed by condensation of glycoluril with formaldehyde under acid conditions, which are the fourth macrocyclic hosts after crown ethers, cyclodextrins, and calixarenes [Figure 1C][115-117]. According to the different number of glycoluril units, different types of CB[*n*]s can be obtained, and common cucurbiturils include CB[6], CB[7], and CB[8][118,119]. Due to their unique structures of hydrophobic cavity and hydrophilic port, CB[*n*]s are easy to form host-guest complexes with drug molecules and are promising materials for reducing side effects and enhancing the stability of antitumor drugs[120-124]. In addition, CB[*n*]s-based supramolecular nanosystems can be used to effectively reverse cancer drug resistance[125-128].

Cancer drug resistance is closely related to the inhibition of tumor apoptosis[129,130]. Mitochondria serves as the center for regulating tumor cell apoptosis[131,132]. Therefore, the destruction of mitochondria is also an effective way to overcome cancer drug resistance[133-135]. Recently, Dai *et al.* synthesized a multivalent supramolecular polymer (HABMitP) by modifying HA with mitochondrial targeting peptide and 4-bromophenylpyridium [Figure 9A][136]. The combination of HABMitP, cisplatin, and CB[8] could promote mitochondrial aggregation, which led to the deterioration of mitochondria to release apoptosis-inducing factor (cytochrome C), thereby activating the apoptosis of tumor cells [Figure 9B and C]. Moreover, cisplatin-resistant tumors did not grow treated with CisPt + HABMitP + CB[8] for 14 days, indicating that assembly-induced mitochondrial aggregation significantly improved the antitumor efficacy of cisplatin [Figure 9D]. This study showed that the regulation of mitochondrial behavior was beneficial to the reversal of drug resistance, which provided a broad prospect for overcoming tumor drug resistance.

The inhibition of P-gp expression by reducing ATP concentration can reduce drug resistance in tumor cells[137,138]. Wang *et al.* reported nanoparticles (SCC-NPs) based on CB[7], which encapsulated the anticancer drug oxaliplatin (OxPt) and mitochondria-targeting peptide (N-Phe-KLAK) by the excellent host-guest properties of CB[7] [Figure 10A][139]. Due to the special acid responsiveness and competitiveness of the polymeric shell, SCC-NPs were used for self-motivated supramolecular combination chemotherapy. In acidic tumor environments, the amidomethyl phenylamine moieties on the polymeric shell were restored to form host-guest complexes with CB[7], competing to replace and release OxPt and N-Phe-KLAK. The released N-Phe-KLAK could effectively inhibit the production of ATP, resulting in the damage of energy-dependent drug efflux pump [Figure 10B]. Additionally, the accumulation of OxPt in cells directly led to an increase in the number of apoptotic cancer cells, which successfully inhibited the viability of drug-resistance cells [Figure 10C and D). Self-motivated supramolecular combination chemotherapy provided a new strategy for addressing the issue of cancer drug resistance.

The *Fusobacterium nucleatum* (*F. nucleatum*) with apoptosis-inhibiting effect can trigger drug resistance in colorectal cancer (CRC) cells[140-143]. To address this issue, Yan *et al.* constructed a CB[7]-based nanomedicine (PG-Pt-LA/CB[7]) by multiple assemblies to overcome drug resistance [Figure 11A][144]. PG-Pt-LA/CB[7] targeted and penetrated cancer cells and released OxPt in response to the GSH. The efficient uptake and stable release of drugs increased the accumulation of OxPt in CRC cells. In addition, PG-Pt-LA/CB[7] showed the best inhibition effect on *F. nucleatum* compared to OxPt and PG-Pt-LA, successfully overcoming the drug resistance of CRC cells caused by *F. nucleatum* [Figure 11B]. A negligible growth in tumor volume was observed after 18 d of incubating tumors with PG-Pt-LA/CB[7], showing that PG-Pt-LA/CB[7] improved the chemotherapeutic effect of OxPt on CRC cells [Figure 11C]. PG-Pt-LA/CB[7] was

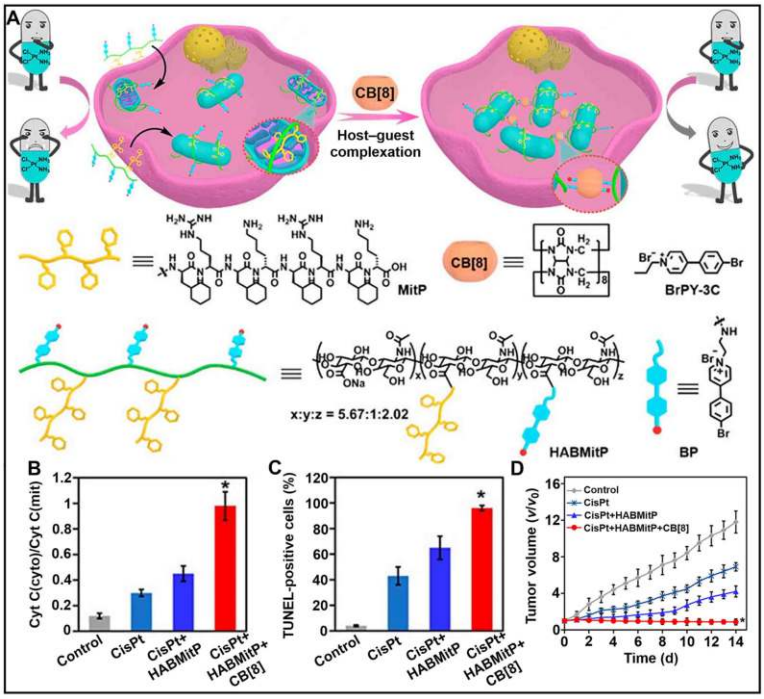


Figure 9. (A) Schematic illustration of mitochondrial aggregation progress after treatment with multivalent supramolecular polymer (HABMitP) and CB[8]; (B) The ratio of cytosol cytochrome C [Cyt C(cyto)] to mitochondrial cytochrome C [Cyt C(mit)] and (C) The apoptosis percentage of TUNEL-positive cells with different treatments; (D) Changes of tumor volume in tumor-bearing mice with different formulations ($^{*}P < 0.05$). This figure is quoted with permission from Dai *et al.*[136].

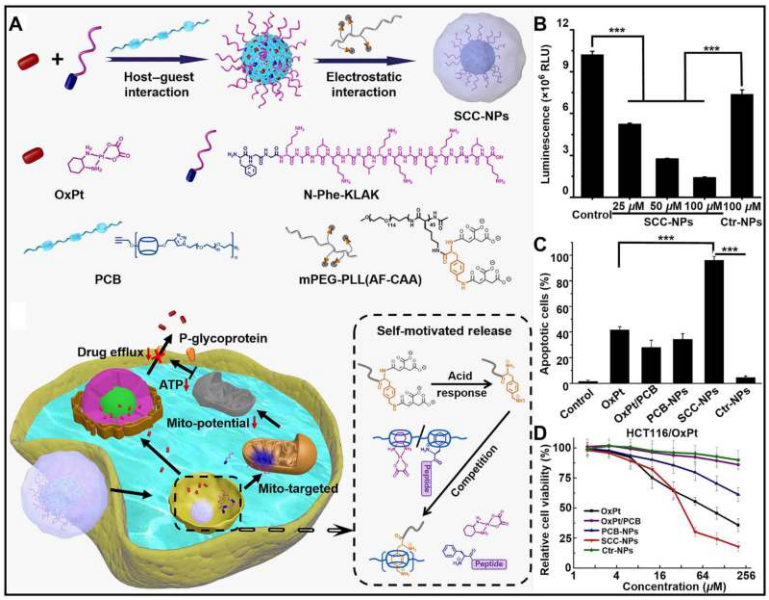


Figure 10. (A) Schematic illustration of the preparation and mechanism of self-motivated nanoparticles (SCC-NPs) in overcoming drug resistance; (B) ATP levels in HCT116/OxPt cells treated with Ctr NPs and SCC NPs at different doses; (C) The number of apoptotic cells treated with different formulations; (D) Cell viability of HCT116/OxPt cells after incubating with different treatments ($^{***}P < 0.001$). This figure is quoted with permission from Wang *et al.*[139]. PEG: Poly (ethylene glycol).

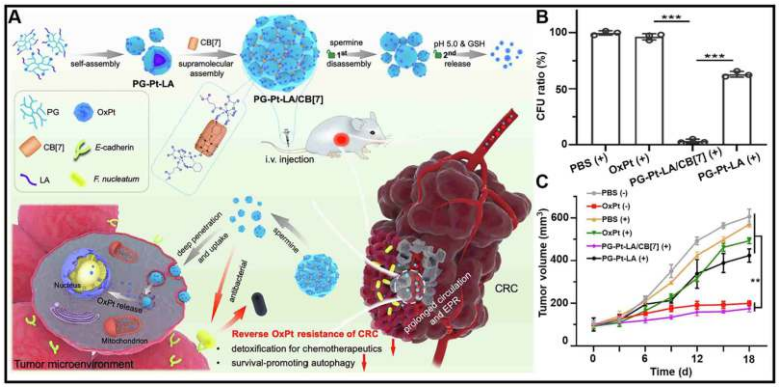


Figure 11. (A) Schematic illustration of the preparation and mechanism of CB[7]-based nanomedicine (PG-Pt-LA/CB[7]) in overcoming drug resistance of CRC cells; (B) Changes of *F. nucleatum* levels in CRC cells with different treatments; (C) Changes of tumor volume in tumor-bearing mice with different formulations ($^{**}P < 0.01$, $^{***}P < 0.001$). This figure is quoted with permission from Yan *et al.*[144]. CRC: Colorectal cancer.

expected to be a good material for improving the effect of OxPt on CRC cells.

PILLARARENES-BASED HOST-GUEST NANOSYSTEMS FOR OVERCOMING CANCER DRUG RESISTANCE

Pillararenes (P[*n*]As) are a new type of macrocyclic hosts that bridge hydroquinone units through methylene discovered by Ogoshi *et al.* [Figure 1D][145]. These hydroquinone units are generally 5-10, with P[5]A and P[6]A being the most common[146-151]. P[*n*]As are widely used in various fields such as drug delivery, ion recognition, adsorptive separation, sensors, and optoelectronic materials due to the characteristics of symmetrical rigid skeleton, adjustable electron-rich cavity, and easy functionalization[152-154]. In addition, because of the highly attractive host-guest properties of P[*n*]As, more and more attention has been paid to the construction of P[*n*]As-based host-guest nanosystems to overcome cancer drug resistance[155-157]. Liu *et al.* prepared a novel carboxylatopillar[5]arene-based supramolecular quaternary ammonium nanoparticle to overcome the drug resistance generated during the chemotherapy of CRC[158]. Chang *et al.* constructed a redox-responsive cationic vesicle based on amphiphilic pillar[5]arene, successfully overcoming the drug resistance of tumors[159].

The water-soluble pillar[6]arene (WP6) not only forms stable host-guest complexes with a variety of guest molecules but also exhibits good biocompatibility and stimuli-responsiveness, which offers the possibility for constructing supramolecular host-guest nanoplatforms to reverse cancer drug resistance[160-166]. Shao *et al.* reported a host-guest complex (AWP6⊃G) containing anionic WP6 (AWP6) and prodrug (G), which further self-assembled to form nanovesicles for inhibiting cancer drug resistance [Figure 12A][167]. The nanovesicles released camptothecin (CPT) and chlorambucil (Cb) under the action of GSH to achieve combination chemotherapy. The dual-drug co-loaded nanovesicles showed a better inhibition effect on drug-resistance cells compared to the single drug [Figure 12B]. This study showed that P[*n*]As-based supramolecular host-guest nanosystems were expected to be ideal materials for inhibiting MDR.

Subsequently, Liu *et al.* prepared a nanosponge (NS) based on AWP6 using a "bottom-up" template preparation technique [Figure 12C][168]. Through the host-guest interaction, antitumor drugs and dyes were stably encapsulated in AWP6 to overcome MDR. The IC_{50} of DOX@NS (3.4 μM) was significantly lower than that of free DOX (34.4 μM) when different doses of free DOX and DOX-loaded NS were incubated in drug-resistance cells [Figure 12D]. Mechanistic studies indicated that the effective loading and stable

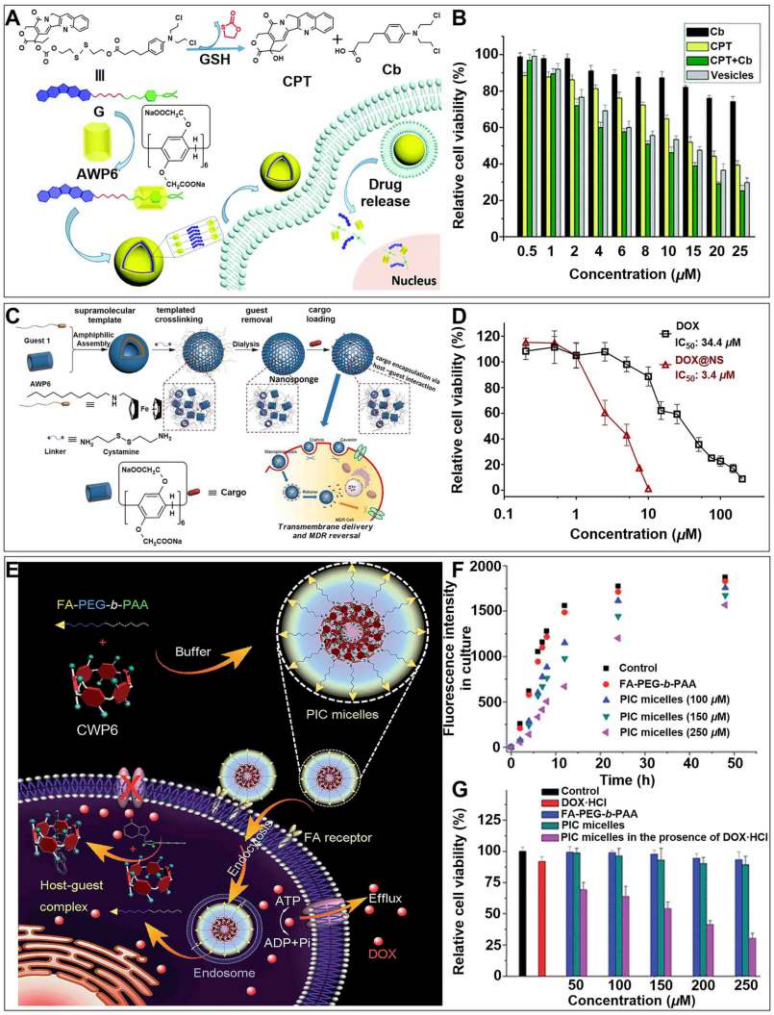


Figure 12. (A) Schematic illustration of the formation of nanovesicle and its internalization progress; (B) Cell viability of MCF-7 cells after incubation with Cb, CPT, Cb + CPT mixture, and vesicles for 24 h. This figure is quoted with permission from Shao *et al.*[167]; (C) Schematic illustration of water-solution pillar[6]arene nanosponges (NS) in overcoming MDR; (D) Cell viability of MCF-7/ADR cells after incubation with DOX and DOX@NS. This figure is quoted with permission from Liu *et al.*[168]; (E) Schematic illustration of the preparation of PIC micelles and their application in inhibiting drug e ux; (F) Changes of extracellular fluorescence intensity after incubating with FA-PEG-*b*-PAA and di erent concentrations of PIC micelles; (G) Cell viability of MCF-7/ADR cells after incubation with different treatments. This figure is quoted with permission from Yu *et al.*[170]. AWP6: Anionic WP6; CPT: camptothecin; DOX: doxorubicin; FA: folic acid; GSH: glutathione; MDR: multidrug resistance; NS: nanosponge; PEG: poly (ethylene glycol); PIC: polyion complex.

encapsulation of DOX based on host-guest interaction were the main reasons for overcoming MDR. This work showed that the delivery of anticancer drugs through host-guest interaction was a promising way to overcome MDR.

Additionally, cationic WP6 (CWP6) can encapsulate ATP, blocking the energy of drug efflux[169]. Yu *et al.* prepared a polyion complex (PIC) micelle by modifying CWP6 with functionalized diblock copolymer (FA-PEG-*b*-PAA) [Figure 12E][170]. PIC micelles could specifically target and penetrate cancer cells overexpressed with FA receptors. The decrease in extracellular fluorescence intensity indicated that CWP6 successfully blocked the energy source of calcein (model drug) efflux [Figure 12F]. In addition, PIC micelles significantly enhanced the inhibitory effect of DOX·HCl on cell viability compared with free DOX·HCl [Figure 12G]. These results suggested that the supramolecular nanomicelle endocytosed by drug-resistance cells and

released CWP6 to selectively form a host-guest complex with ATP, which provided a new method for blocking the energy of drug efflux and was expected to become an ideal material for overcoming cancer drug resistance.

In addition to blocking the energy source of P-gp expression, nitric oxide (NO) can also downregulate the expression level of P-gp, reversing the drug resistance of cancer[171-173]. To achieve stable delivery and selective release of NO in tumor cells, Ding *et al.* designed supramolecular peptide nanomedicine (BPC/DOX-ICG) based on the host-guest complexation of anionic water-soluble [2]biphenyl-extended-pillar[6]arene (AWBpP6) with pyridinium-terminal-modified polypeptide (PPNC) [Figure 13A][174]. DOX and indocyanine green (ICG) loaded in BPC/DOX-ICG were used to simultaneously treat cancer cells with chemotherapy and photothermal therapy. *S*-nitrosothiol on PPNC released NO to downregulate the expression level of P-gp after near-infrared (NIR) irradiation. Western Blot analysis showed that the P-gp level in MCF-7/ADR cells was significantly reduced to 24.9% when treated with NIR irradiation and BPC/DOX-ICG [Figure 13B]. The reduced P-gp could greatly enhance the efficacy of chemotherapeutic drugs, inhibiting tumor growth [Figure 13C]. Therefore, P[*n*]As-based nanocarriers could effectively deliver NO to downregulate P-gp expression, providing a promising approach to eliminate cancer drug resistance.

Chloride channel protein is highly expressed in various cancer cells and has a significant correlation with tumor drug resistance[175,176]. Yang *et al.* reported a supramolecular nanoprodrug (DOX@GP5⊃Pro-NFA) based on the host-guest complexation between galactose-modified pillar[5]arene (GP5) and chloride channel inhibitor prodrug (Pro-NFA) to reverse drug resistance [Figure 14A][177]. DOX@GP5⊃Pro-NFA was hydrolyzed under the action of esterase to release DOX and NFA, which could effectively block chloride ion channels, and reverse cancer drug resistance. Additionally, the inhibitory effect of DOX@GP5⊃Pro-NFA on tumor cells, especially drug-resistance cells, was significantly higher than that of free DOX [Figure 14B]. Moreover, poly(ADP ribose)polymerase (PARP) can repair DNA to directly lead to drug resistance[178,179]. Yang *et al.* designed a nanoparticle (DOX@GP5⊃Pro-ANI) based on GP5 to load PARP inhibitor prodrug (Pro-ANI) that could inhibit DNA repair [Figure 14C][180]. DOX@GP5⊃Pro-ANI overcame tumor drug resistance by inhibiting the expression of PARP, effectively reducing the viability of drug-resistance cells [Figure 14D]. These studies showed that P[*n*]As could effectively load anticancer prodrugs, which opened up broad prospects for inhibiting the expression of proteins associated with drug resistance.

CONCLUSION

In summary, we reviewed the application of supramolecular host-guest nanosystems based on cyclodextrins, calixarenes, cucurbiturils, and pillararenes in overcoming cancer drug resistance. Compared with traditional small molecule drugs, nanosystems can effectively reduce the side effects of drugs and improve the accumulation of drugs in tumors. However, traditional nanosystems also have some drawbacks, such as lack of stimuli-responsiveness, difficulty in preparation and synthesis, and slow degradation *in vivo*. The emergence of supramolecular nanosystems complements the drawbacks of these traditional nanosystems. Due to their dynamic and reversible host-guest interactions, supramolecular nanosystems are endowed with rich stimuli-responsiveness to release drugs within tumors. Furthermore, supramolecular host-guest nanosystems have some advantages, such as high drug loading, low side effects, and good biocompatibility, which can co-deliver multiple drugs to inhibit cancer drug resistance by damaging mitochondrial function, blocking the energy source, inhibiting DNA repair, and reducing the level of GSH. However, supramolecular host-guest nanosystems still face some challenges in overcoming cancer drug resistance:

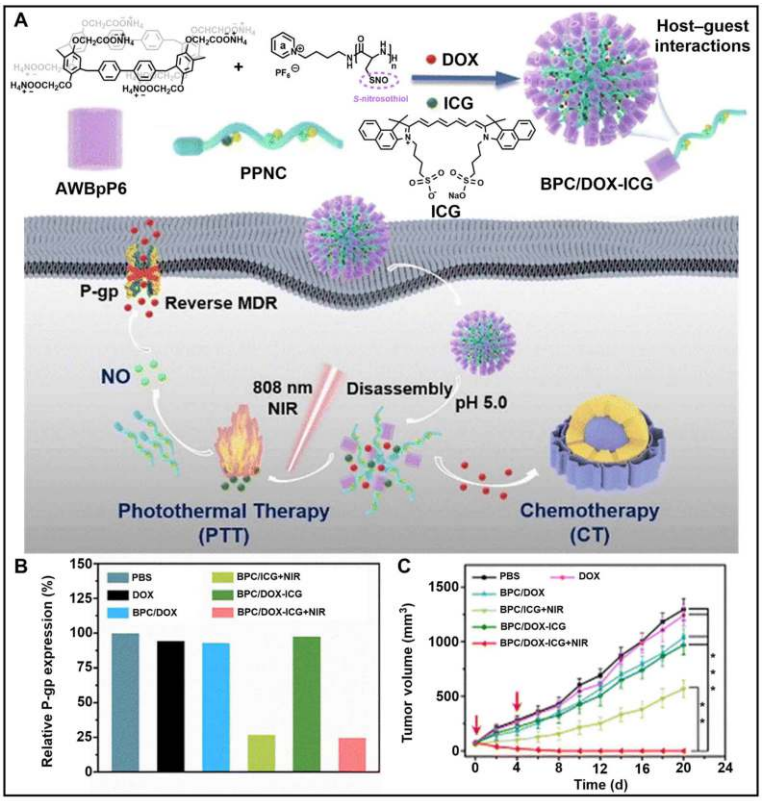


Figure 13. (A) Schematic illustration of synergistic PTT and CT using supramolecular polypeptide nanomedicine (BPC/DOX-ICG); (B) The P-gp expression levels in MCF-7/ADR cells with different treatments; (C) Changes of tumor volume in tumor-bearing mice with different formulations ($^{**}P < 0.01$, $^{***}P < 0.001$). This figure is quoted with permission from Ding *et al.*[174]. DOX: Doxorubicin; ICG: indocyanine green; MDR: multidrug resistance; NIR: near-infrared; PPNC: pyridinium-terminal-modified polypeptide.

(i) Further improve the targeting. Tumor cells differ from healthy cells in many ways, such as acidity, hypoxia, and metabolism. Thus, more specific supramolecular host-guest nanosystems should be developed by focusing on the tumor microenvironment to target and penetrate tumor cells, reversing cancer drug resistance caused by drug uptake;

(ii) Optimize drug loading strategy. Although multidrug-loaded supramolecular host-guest nanosystems can inhibit drug resistance, their effects are difficult to predict due to the different pharmacokinetics of each drug. Therefore, it is necessary to optimize the combination and dosage of loaded drugs and design supramolecular host-guest nanosystems with excellent performances;

(iii) Improve stability. Supramolecular host-guest nanosystems have rich stimuli-responsiveness, but at the same time, there are problems of poor stability. In future studies, the stimuli-responsiveness of supramolecule and the stability of macromolecule can be better combined to prepare supramolecular polymeric nanosystems to overcome cancer drug resistance;

(iv) Promote the development of cancer synergistic therapy. At present, most supramolecular host-guest nanosystems used to overcome drug resistance of tumors remain at the level of drug delivery, which greatly limits the inhibitory effect on drug-resistance cells. Introducing other therapeutic methods (such as photodynamic therapy, gene therapy, and immunotherapy) into supramolecular host-guest nanosystems will help establish more accurate and personalized strategies to combat cancer drug resistance;

(v) Enhance the clinical translation. While some preliminary studies have shown that supramolecular host-guest nanosystems can be used to overcome drug resistance in cancer, there is still a long way to go before clinical translation. Firstly, the pharmacokinetics, biodistribution, metabolic behavior, and toxicological

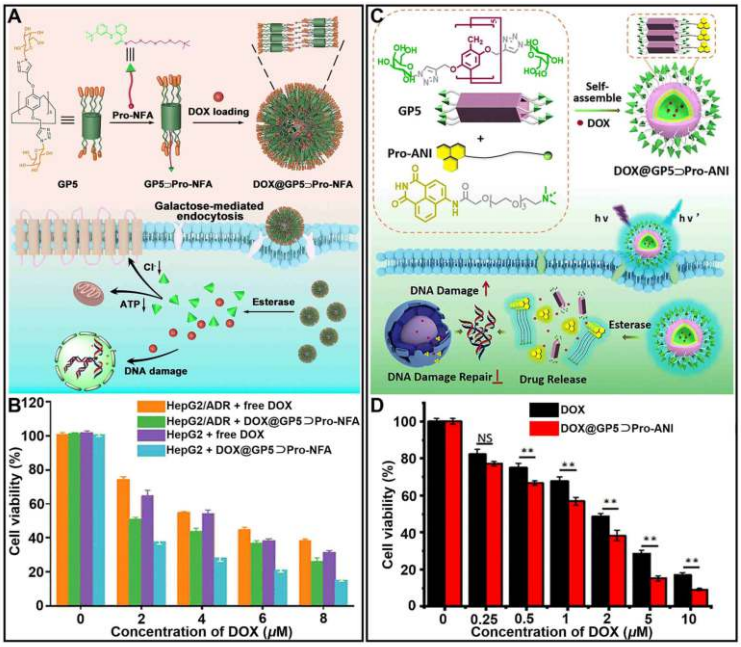


Figure 14. (A) Schematic illustration of the preparation of supramolecular nanoprodrugsDOX@GP5⊃Pro-NFA and their applications in overcoming cancer drug resistance; (B) Cell viability of HepG2 cells and HepG2/ADR cells treated with free DOX and DOX@GP5⊃Pro-NFA, respectively. This figure is quoted with permission from Yang *et al.*[177]; (C) Schematic illustration of the preparation of supramolecular nanoprodrugs DOX@GP5⊃Pro-ANI and their applications in overcoming cancer drug resistance; (D) Cell viability of HepG2/ADR cells treated with free DOX and DOX@GP5⊃Pro-ANI ($^{**}P < 0.01$). This figure is quoted with permission from Yang *et al.*[180]. DOX: Doxorubicin; GP5: galactose-modified pillar[5]arene.

characteristics of supramolecular host-guest nanosystems are still in the research stage, and there are still unpredictable risks to their safety. Secondly, *in vitro* and *in vivo* experiments of supramolecular host-guest nanosystems cannot completely mimic the complex microenvironment of tumors in the body, resulting in lower clinical therapeutic effects than expected. Thirdly, the large-scale production of supramolecular host-guest nanosystems is a bottleneck in clinical applications, and small changes in the manufacturing process can cause significant changes in their physicochemical properties, which will affect their safety and biological effects. Therefore, more basic research and clinical trials are needed to assess their safety, efficacy, and feasibility.

The development of supramolecular host-guest nanosystems offers new hope to alleviate drug resistance in cancer, although innovation and progress are still required in many aspects. We believe that with continued research efforts, supramolecular host-guest nanosystems will make further progress in reversing cancer drug resistance, and bring new breakthroughs for cancer treatment and even human health.

DECLARATIONS

Authors' contributions

Conceptualization: Wu S, Zhou J
Original draft preparation: Wu S
Review and editing: Zhou J, Yan M, Liang M, Yang W, Chen J
Supervision: Zhou J

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Targeting BCL2 pathways in CLL: a story of resistance and ingenuity

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Abstract

Chronic lymphocytic leukemia (CLL) is common amongst leukemic malignancies, prompting dedicated investigation throughout the years. Over the last decade, the treatment for CLL has significantly advanced with agents targeting B-cell lymphoma 2 (BCL2), Bruton's tyrosine kinase, and CD20. Single agents or combinations of these targets have proven efficacy. Unfortunately, resistance to one or multiple of the new treatment targets develops. Our review investigates various mechanisms of resistance to BCL2 inhibitors, including mutations in BCL2, alterations in the Bcl protein pathway, epigenetic modifications, genetic heterogeneity, Richter transformation, and alterations in oxidative phosphorylation. Additionally, the review will discuss potential avenues to overcome this resistance with novel agents such as bispecific antibodies, Bruton's tyrosine kinase (BTK) degraders, non-covalent BTK inhibitors, and chimeric antigen receptor T (CART).

Keywords: BCl-2 inhibitors, apoptosis, CLL, resistance, tumor microenvironments, cell cycle regulation, genetic mutations, epigenetics, richter transformation

INTRODUCTION

Chronic lymphocytic leukemia (CLL) has been the most common form of Leukemia in the developed world for the last decade, according to the Surveillance epidemiology and end result database[1]. Treatment and,

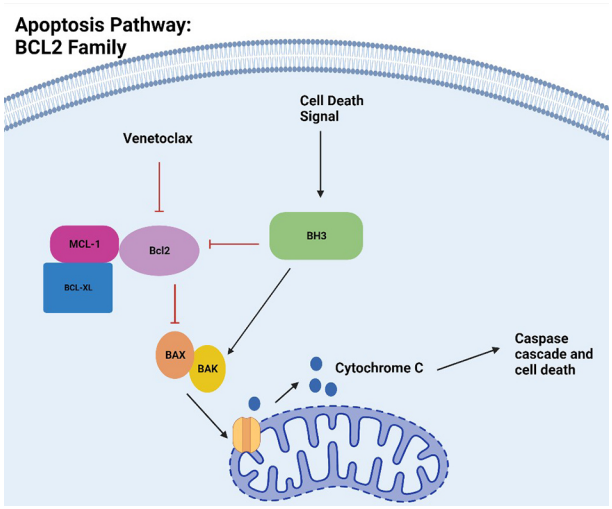

Figure 1. Apoptosis Pathway: BCL2 Proteins. Made with Bioreader with data from the following publications: Roy *et al.*[11]; Youle *et al.*[12]. BCL2: B-cell lymphoma 2.

therefore, overall prognosis have improved significantly during this time. Investigation into the pathophysiology of CLL allowed for the development of targeted agents, including Burton's tyrosine kinase (BTK) inhibitors, anti-CD20 monoclonal antibody, and B-cell lymphoma 2 (BCL2) inhibitors[2]. Ibrutinib, a BTK inhibitor, proved to be an effective treatment of CLL in the first line[3]. Venetoclax, a BCL2 inhibitor, was first utilized in relapsed disease alone and then in combination with rituximab [Table 1][4-10]. More recently, the combination of ibrutinib and venetoclax was approved for front-line treatment of CLL in Europe after findings from the GLOW trial [NCT03462719] and CAPTIVATE trials [NCT02910583][6,7] [Table 1].

Widespread use of venetoclax in hematologic malignancies prompted further research into the BCL22 apoptosis pathway, allowing for the identification of the key agents involved. From extensive research, we have found that in non-cancerous cells, after receiving a pro-apoptotic signal, the BH3-only proteins will activate additional proteins, BAX and BAK, by binding directly or by binding to anti-apoptotic proteins, BCL2, BCL-XL, MCL-1, thereby freeing these pro-apoptotic proteins to travel to the mitochondrial membrane forming pores, releasing cytochrome c which stimulates the caspase cascade for apoptosis[11,12] [Figure 1]. Venetoclax promotes apoptosis by binding to BCL2, enabling the release of the pro-apoptotic proteins to trigger apoptosis[13] [Figure 1]. Unfortunately, resistance to venetoclax develops by several distinct mechanisms, including mutations in BCL2, epigenetic pathways, alterations in oxidative phosphorylation, alterations in BCL2 pathway, tumor microenvironment, genetic heterogeneity, and Ritcher's transformation. We will discuss each of these mechanisms, focusing on the contributions to resistance in this review. Additionally, we will propose various methods to overcome the various resistance pathways.

BCL2 INHIBITOR RESISTANCE MECHANISMS

Genetic mutations in BCL2

Alterations in the substrate or target thereby conferring resistance is a common theme in biology and venetoclax resistance is no exception. A recent analysis of CLL patients who progressed on venetoclax found that 7 of the 15 patients developed a mutation, Gly101Val, in BCL2, which decreases the affinity of BCL2 for venetoclax by overcrowding the BH3 binding groove, thereby preventing venetoclax from displacing the pro-apoptotic proteins [Table 2][14-18]. Of note, the mutation was not detected prior to starting treatment but

Table 1. Venetoclax trials in CLL

Drug	Line of treatment	Target	Trial	Duration of treatment	Rate of Richter's transformation
Venetoclax + Ibrutinib	First line	BCL2 + BTK	GLOW[6]	Fixed	3 patients (2.8%) *vs.* 2 patients (1.9%) in control arm
Venetoclax + Ibrutinib	First line	BCL2 + BTK	CAPTIVATE[7]	Fixed	Not documented
Venetoclax + Ibrutinib	Relapsed/Refractory	BCL2 + BTK	CLARITY[8]	Fixed	0 patients
Venetoclax	Refractory, 17p mutated	BCL2	Phase II[9]	Till progression	11 patients (10.3%)
Venetoclax + Rituximab	Refractory	BCL2 + CD20	MURANO[5]	Fixed	6 patients (3.1%) *vs.* 5 patients (2.65%) in control arm
Venetoclax + Obinutuzumab	First line	BCL2 + CD20	CLL14[10]	Fixed	2 patients (0.94%) *vs.* 1 patient (0.46%) in control arm

BCL2: B-cell lymphoma 2; BTK: Bruton's tyrosine kinase; CLL: chronic lymphocytic leukemia.

Table 2. Venetoclax resistance: mutations in BCL2

Mutation	Mutation type	Mutation site	Found in	Frequency in patients
Gly101Val[14]	Point	BH3 binding groove	CLL	7/15 (46.6%)
Phe104Ile[16]	Point	BH3 binding groove	Follicular lymphoma	1/1 (100%)
Gly101Ala[18]	Point	BH3 binding groove	CLL	1/11 (9%)
Asp103Tyr[15]	Point	BH3 binding groove	CLL	1/4 (25%)
Ala113Glu[18]	Point	Non-binding	CLL	1/11 (9%)
Phe104Cys[17]	Point	BH3 binding groove	Murine human-like MCL cell lines	NA
Phe104Leu[17]	Point	BH3 binding groove	Murine human-like MCL cell lines	NA
Leu119Val[18]	Point	Unknown	CLL	1/11 (9%)
Arg107_Arg110[18]	Frame shift	Unknown	CLL	3/11 (27.3%)

BCL2: B-cell lymphoma 2; CLL: chronic lymphocytic leukemia.

rather was detected after 19-42 months of treatment[14]. Mutations that also confer resistance due to the impact on binding include Phe104Ile located at the venetoclax binding site of BCL2 and Asp103Tyr, an essential part of hydrogen binding of venetoclax which have been identified in follicular lymphoma and CLL, respectively[15,16] [Table 2]. In mantle cell lymphoma cell lines, Phe104Cys and Phe104leu missense mutations have also been found to alter the BH3 domain and therefore binding affinity[17] [Table 2]. From a retrospective analysis of CLL patients whose disease was refractory to ibrutinib and resistant to venetoclax, multiple mutations including point mutations in Gly101Ala, Ala113Gly, Leu119Val, Asp113Glu and in-frame insertion of Arg107_Arg110 were observed[18] [Table 2]. Of note, the BCL2 mutations were noted to be sub-clonal with a varying percentage of cells (from 7%-70%, vast majority < 50%), indicating multiple resistance patterns are likely involved[14-18]. This finding would argue against these mutations representing so-called "driver mutations", but there is not enough evidence to definitively determine this. As the general CLL population is not tested for the above mutations given their rarity, it is impossible to give an overall frequency. From the original study identifying the G101V mutation, 21 out of 67 patients had progression on venetoclax, of which 15 samples were analyzed and roughly 50% (7 patients) developed the mutation after venetoclax as the mutation was not present prior[14]. With further analysis into venetoclax-resistant patients, additional mutations conferring various changes in the structure of the BCL2 will likely be identified and methods to overcome these mutations will follow.

Epigenetic modifications

For the last decade, scientists investigated modifications of translation with gene activation or deactivation and the corresponding downstream effects. In the case of the BCL2 pathway, these epigenetic alterations may play a larger role in resistance than direct mutations in the BCL2 protein. A recent study used advanced molecular techniques including CRISPR, whole-exome sequencing, and methylated DNA immunoprecipitation sequencing to identify a regulatory CpG island within the PUMA (a BH3-only protein) promoter site which was shown to be methylated and therefore silenced gene expression (favoring oxidative phosphorylation and cell survival) after the administration of venetoclax, indicating resistance[19]. This data was obtained from both CLL patients (6 patients) and VEN/S63845 resistant cell lines. Further proof of this concept was demonstrated by the restoration of venetoclax function (cell death) after inhibition of methyltransferases[19].

Non-coding RNAs, including microRNA (miRNA) and long non-coding RNA (lncRNA), have been investigated extensively in the last two decades in CLL due to their involvement in cell cycle regulation among other cellular mechanics, thereby promoting resistance[20-22]. Additionally, RNA cytosine methyltransferases NSUN1 and NSUN2 have been shown to induce venetoclax resistance in leukemic cells via interactions with RNA polymerase II extension complex, knocking down NSUN1 or NSUN2 returned sensitivity to venetoclax[23]. Much is still not fully understood regarding these complex epigenetic regulations, as this is an area of future research and investigation.

Alterations in BCL2 pathway

As the BCL2 pathway is complex, alterations or upregulation of other components have also been the subject of investigation. Mutations in the effector proteins BAX/BAK may be venetoclax specific as one analysis found mutations in BAX followed venetoclax treatment in 30% of the patients but not after treatment with ibrutinib[24]. Further, a mutation in the C terminal transmembrane domain (G179E) of BAX prevents the anchoring of BAX to Mitochondria, thereby blocking venetoclax-induced apoptosis[17,25].

In a 2011 study of the novel agent ABT-737, which inhibits BCL2, BCL-XL, and BCL-w, the levels of MCL-1 and BFL-1 were significantly higher than BCL2 in the population resistant to the drug, while the sensitive population had the highest levels of BCL2 comparatively[26]. Conversely, in some tumor models, cells express low levels of BCL2 but are still highly sensitive to BCL-2 inhibition, indicating that the BCL2 protein is a small part of a more intricate process[17]. Other anti-apoptotic proteins, such as MCL-1 and BCL-XL, are not directly inhibited by venetoclax but appear to have a role in resistance, with BCL-XL appearing to have the strongest impact[27]. The overexpression of BCL-XL is associated with venetoclax resistance and the upregulation of NF-kB signaling (cell survival); the addition of BCL-XL inhibitors can restore cell sensitivity to venetoclax[27].

Likewise, MCL-1, involved in the sequestration of BIM and binding of BAK which prevents apoptosis is commonly overexpressed in venetoclax-resistant patients[13,28]. MCL-1 has been the subject of interest as there are various efforts at utilizing its inhibition as a potential therapeutic option, occasionally in conjunction with venetoclax[29]. Yet still, the matter is more complicated as additional proteins involved in this pathway were also found to have interactions with MCL-1 and BCL-XL, namely BFL-1[30]. Research into the detailed interactions between the pro-apoptotic and anti-apoptotic protein signaling balance is warranted, as any number of these proteins could be targeted for treatment.

Tumor microenvironment

For added complexity, tumor microenvironment including alterations in cell metabolism and signaling may also contribute to resistance. When CLL and MCL cells were preincubated with anti-apoptotic/pro-growth

signaling factors from outside the direct BCL2 pathway, namely sCD40L, IL-10, CpG-ODN, B-cell-activating factor (BAFF), CXCL3, the combination of sCD40L, IL-10, and CpG-ODN had the lowest level of ibrutinib/venetoclax induced cytotoxicity indicating resistance[31]. Even high levels of ibrutinib and venetoclax did not achieve an adequate level of cytotoxicity, but when NF-kB signaling was inhibited by the addition of proteasome inhibitors, bortezomib and carfilzomib, sensitivity to ibrutinib/venetoclax returned[31]. Further research into this topic may reveal potential therapeutic targets for patients experiencing relapse/resistance.

Genetic heterogeneity

The extensive variation in the genetics of CLL patients has been noted, and the most common alterations include deletions of chromosomes 13q, 11q, 17p, and trisomy 12[32]. This heterogeneity may also play a role in resistance, as seen with the 17p deletion, which is not only associated with advanced disease/poor prognosis but also correlated with resistance, as one study found 7 out of 11 venetoclax-resistant CLL patients harbored the *TP53* aberration[18,32]. Further, trisomy 12 contributes to increased expression of MCL-1 which also has been associated with venetoclax resistance[33]. In addition to chromosomal alterations, other genetic alterations have been associated with resistance; in a study of 8 venetoclax resistance patients, 2 patients were found to have potential targetable mutations (BRAF and PD-L1), both thought to be involved in MCL-1 upregulation[34]. Additionally, a homozygous mutation in *CDKN2A/B*, a cell cycle regulator, was also identified in the resistant patient population[34]. A combined analysis of several CLL studies found *TP53*, *SF3B1*, *MYD88*, *NOTCH1*, and *ATM* were the most mutated genes with varying rates of mutation across the studies[32]. Interestingly, there was variation across the mutations, as certain mutations arise continuously throughout disease (*TP53*, *ATM*), others arise after treatment initiation (*NOTCH1*), while others remain in the same frequency throughout the disease course (*MYD88*)[35].

Abnormal oxidative phosphorylation

As the BCL2 pathway ultimately involves mitochondria, there has been consideration of the role of oxidative phosphorylation in resistance. In other cancerous cell lines, increased levels of oxidative phosphorylation and reactive oxygen species are associated with resistance to chemotherapeutic agents[36]. Investigation into CLL cell lines in vitro found that the resistant cells had significantly higher levels of both basal and maximal oxygen consumption from ATP production by oxidative phosphorylation as well as increased mitochondrial membrane potential[37]. Additionally, after the cell lines were treated with venetoclax, a decline in oxygen consumption was observed, but this was dependent on the ability of pore formation (BAX/BAK) as knockout cell lines did not have the same response to venetoclax[37]. Additional research into cell metabolism may further elucidate details regarding these complex interactions and potential treatment targets.

Richter transformation

Transformation of CLL contributing to venetoclax resistance is one of the less well-studied mechanisms of resistance. Recent analysis has shown that increased genetic instability during transformation can result in the development of mutations related to venetoclax resistance[27]. While the more common BCL2 mutation, Gly101Val, was not seen in the transformed population, a rarer mutation, Arg110dup, was seen in a low percentage (< 0.5%)[18]. Rates of Richter's transformation vary by trial but were generally low [Table 1]. Currently, data on Richter transformation in CLL remains limited, but it is an area of ongoing investigation.

METHODS TO OVERCOME RESISTANCE

While the first action after resistance to treatment is to alter treatment to another agent, researchers have identified several other avenues to combat resistance, including other formulations of bcl2 inhibitors, chimeric antigen receptor T (CART), BTK degraders, non-covalent BTK inhibitors, phosphoinositide 3-

kinase inhibitors, and novel bispecific antibodies. Additionally, duration of treatment, fixed *vs.* continuous, may be instrumental in the development of resistance. An investigation into relapsed CLL patients treated with venetoclax and rituximab followed by venetoclax monotherapy found that among the durable responses (33 patients of which 14 remained on monotherapy and 19 stopped venetoclax), five-year estimates of ongoing response rate were similar, 71% (95%CI, 39-88) in continuous treatment *vs.* 79% (95%CI, 49-93) in the fixed duration group[38]. However, an analysis of single-agent venetoclax in CLL patients with prolonged follow-up found ongoing venetoclax treatment may be a driver of resistance, as activation of NF-kB with associated MCL1 expression was increased in all relapsed samples while on venetoclax therapy compared to off therapy[39]. This concept is further supported by a patient who achieved minimal residual disease on venetoclax with fixed treatment duration, and did not have increased NF-kB or other cell survival signaling[39]. Further, analysis of the MURANO trial with fixed duration combination treatment did not identify any mutations in *BCL2*, a known mechanism of resistance as discussed above[40].

BCL2 inhibitors/BH3 mimetic

Since the discovery of the BCL2 family of proteins involved in apoptosis, there has been an evaluation of BCL2-targeted agents. Obatoclax, a BH3 mimetic that antagonizes Mcl-1/Bcl-xL and Bcl-w but not BCL2, was evaluated in a phase I/II with bortezomib in relapsed refractory mantle cell lymphoma, but ORR was modest at 31% with myelosuppression and fatigue as the most common grade 3/4 adverse events[41]. Additionally, navitoclax, another BH3 mimetic, demonstrated 55% ORR when used with Rituximab for 12 weeks and 70% when used with Rituximab continuously until progression or intolerance compared to 35% ORR with rituximab alone in previously untreated CLL patients[42]. Unfortunately, significant thrombocytopenia limited widespread use as it was often dose-limiting[43,44]. Recently, Lisaftoclax, which selectively binds Bcl2 and prevents BCL2:BIM complexes allowing pore formation in mitochondria, demonstrated significant antitumor activity in preclinical trials[45]. This prompted progression to a phase I/II clinical trial in relapsed/refractory CLL patients with an ORR of 65% in the monotherapy group, 98% ORR in combination with acalabrutinib, and 87% in combination with Rituximab[46]. The average number of previous treatments was 2, with 12% of the patients progressing on BTK inhibitors and/or venetoclax[46].

Novel agents

In addition to BH3 mimetics, there has been an investigation into alternative targets with Bispecific antibodies/BiTE. As with venetoclax, the novel agents are explored across B-cell malignancies. Mosunetuzumab, a bispecific T cell engager targeting CD20/CD3, is under evaluation in Non-Hodgkin's lymphoma (NHL) and CLL refractory to at least two lines of treatment in a phase I/II trial [NCT02500407] after promising results of a 60% CR in follicular lymphoma[47]. Other bispecific antibodies targeting CD20/CD3 are currently in various stages of clinical trials, namely odronextamab, glofitamab, epcoritamab, and plamotamab[48-51]. The most common serious adverse event across this drug class remains cytokine release syndrome[48-51]. At this time, these studies consist of mostly large B-cell lymphoma and follicular lymphoma patients, but in the future, the trials could be expanded to include refractory CLL patients.

Since the last decade, the implementation of CART has greatly impacted hematology and the treatment of hematologic malignancies. Initial evaluation of CART in CLL over a decade ago had a small sample size (2 patients), but on long-term follow-up, the patients continued to have a durable remission[52,53]. An analysis of several studies (15 studies, 160 patients) found decreased efficacy of CART in CLL patients with an average CR rate of 30% (0% to 67%) and suggested T-cell dysfunction as a potential rationale for the less robust response[54]. However, a large multicenter study of lisocabtagene maraleucel has recently shown rapid, deep, and durable responses in relapsed/refractory CLL patients after BTKi and venetoclax use[55].

Additionally, there are ongoing clinical trials evaluating novel agents such as BTK degrader (NX-2127) and non-covalent BTK inhibitor (Pirtobrutinib) in relapsed/refractory patients[56,57]. Initial data on pirtobrutinib demonstrated promising results in BTK inhibitor refractory patients, with an ORR of 79% in patients (100 patients) who were refractory to both BTK inhibitors and *BCL2* inhibitors[54]. This success prompted a large phase III trial evaluating pirtobrutinib in the first line in CLL/SLL *vs.* ibrutinib and bendamustine + rituximab[58,59]. The combination of pirtobrutinib and venetoclax is currently in a phase II study in CLL patients in the first line with the primary endpoint of minimal residual disease after 15 cycles [NCT05677919]. Other non-covalent BTK inhibitors such as fenebrutinib and nemtabrutinib have also been investigated, but as both had limited success in phase I trials, their use in B cell malignancies was stopped[60,61]. Phosphoinositide 3-kinase inhibitors have some proven efficacy in CLL, namely idelalisib and duvelisib, with many others under various stages of investigation[62-64]. Combination treatments instead of single-agent therapy may be a method to overcome resistance, as previous CLL studies found that 50% of the patients became refractory to single-agent venetoclax after 2-3 years[4,9].

CONCLUSION

Since the widespread use of venetoclax, more and more has been discovered about intrinsic and extrinsic mechanisms of resistance. Genetic mutations in Bcl-2 are the most common form of venetoclax resistance but have only been reported in approximately 50% of resistant patients, although small sample size (15 patients)[14]. The less well-known forms of resistance, such as epigenetic modifications, alterations of oxidative phosphorylation, and Richter's transformation, may play a larger role in resistance than we know and may become essential in future research of relapsed disease. Given this heterogeneity in resistance in both mechanisms and timeline of development, testing for resistance prior to venetoclax initiation is not warranted. However, there is some data supporting NF-kB expression as a potential biomarker for venetoclax resistance[39], but more investigation is needed to determine its validity prior to widespread application.

Despite the extensive modes of venetoclax resistance, treatment is effective with both monotherapy and combination therapy[4-10]. Even refractory/relapsed CLL patients with poor prognostic factors, like 17p deletion, had durable responses to venetoclax in a phase 2 trial with 54% PFS at 24 months[9]. Interestingly, in a post-hoc analysis of the MURANO trial, dose reduction of venetoclax did not have a significant impact on PFS as long as treatment was not terminated[65]. The implication of dose reduction on resistance was not examined, but it would be beneficial to determine whether lower doses contribute to resistance as this would change management. Additionally, we need further analysis on fixed versus continuous treatment on the development of resistance as initial investigation supports fixed duration treatment in preventing at least certain types of resistance. Research into continuous *vs.* fixed treatment in combination treatments, particularly combination oral agents, could provide clarification.

Novel treatments like bispecific antibodies, BTK degraders, non-covalent binding BTK inhibitors, phosphoinositide 3-kinase inhibitors, and CART may provide the solution for relapsed/refractory patients, but their sequencing order in treatment remains to be determined, especially in CLL where the treated patient population is small. Given the relative novelty of bispecific antibodies, BTK degraders, non-covalent BTK inhibitors, and CART, we do not have long-term data on the impact on resistance. Retrospective analysis of the various clinical trials may provide some insights and should be an area for further research. Regardless of the potential resistance, we can conclude that venetoclax remains a cornerstone in the treatment of CLL.

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Authors' contributions

Made substantial contributions to concept and design as well as editing: Siddiqi T
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Cancer Drug Resistance

Review

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Autophagy-related mechanisms for treatment of multiple myeloma

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Abstract

Multiple myeloma (MM) is a type of hematological cancer that occurs when B cells become malignant. Various drugs such as proteasome inhibitors, immunomodulators, and compounds that cause DNA damage can be used in the treatment of MM. Autophagy, a type 2 cell death mechanism, plays a crucial role in determining the fate of B cells, either promoting their survival or inducing cell death. Therefore, autophagy can either facilitate the progression or hinder the treatment of MM disease. In this review, autophagy mechanisms that may be effective in MM cells were covered and evaluated within the contexts of unfolded protein response (UPR), bone marrow microenvironment (BMME), drug resistance, hypoxia, DNA repair and transcriptional regulation, and apoptosis. The genes that are effective in each mechanism and research efforts on this subject were discussed in detail. Signaling pathways targeted by new drugs to benefit from autophagy in MM disease were covered. The efficacy of drugs that regulate autophagy in MM was examined, and clinical trials on this subject were included. Consequently, among the autophagy mechanisms that are effective in MM, the most suitable ones to be used in the treatment were expressed. The importance of 3D models and microfluidic systems for the discovery of new drugs for autophagy and personalized treatment was emphasized. Ultimately, this review aims to provide a comprehensive overview of MM disease, encompassing autophagy mechanisms, drugs, clinical studies, and further studies.

Keywords: Autophagy, multiple myeloma, unfolded protein response, bone marrow microenvironment, drug resistance, hypoxia, DNA repair and transcriptional regulation, apoptosis

INTRODUCTION

Multiple myeloma (MM) is associated with the proliferation of a single plasma B cell variant in the bone marrow and the secretion of monoclonal immunoglobulins[1]. MM is the second most common type of hematological malignancy worldwide, which accounts for approximately 10% of cases[2]. The mean age of diagnosis is 69 years, and the disease diagnosis is based on the monoclonal M protein produced by malignant B cells[3]. Myeloma cells are plasma cells that secrete immunoglobulins and usually synthesize IgG or IgA[4]. As plasmacytomas increase, monoclonal gammopathy of undetermined significance (MGUS), a premalignant and painless disease, emerges. Subsequently, asymptomatic smoldering myeloma (SMM) and eventually symptomatic MM appear. 1% of cases with MGUS and 10% of cases with SMM might convert to MM[5]. Rapidly accumulating malignant B cells cause bone destruction, anemia, hypercalcemia, and renal failure[6]. In the clinical treatment of the disease, proteasome inhibitors, steroids, monoclonal antibodies, DNA damage, and immunomodulatory compounds are used in combination[7]. Most of these therapies focus on killing MM cells. Commonly, there are three different death mechanisms in cells: apoptosis (type 1), autophagy (type 2), and necrosis (type 3). Apoptosis eliminates irreparable cells and prevents damage from spreading. Nuclear fragmentation, chromatin condensation, and apoptotic bodies commonly crop up in cells during apoptosis[8]. Autophagy is a process of cellular self-degradation that helps the body repair itself by breaking down damaged materials under conditions of starvation or stress. In the autophagic mechanism, gathered proteins in the cell, wrecked organelles or microorganisms are digested in large vesicles with the help of the lysosome[9]. Necrosis is a critical condition that occurs when living tissue is damaged beyond repair, leading to cell death. An exogenous factor such as injury provokes necrosis formation in cells, which causes disruption of cell morphology and damage to organelles[10]. Currently, it was reported that cell death types can be converted into each other through different gene expressions[11]. For example, caspase-8 has a critical function as a bridge between the apoptosis and necrosis pathways[12]. Additionally, inhibition of autophagy was associated with necrosis, inflammation, and accelerated tumor growth[13]. This review extensively delves into the mechanisms of autophagy related to MM disease progression or regression. We clarified how signaling pathways and genes in autophagy mechanisms may affect the disease. Additionally, we elucidate how the drugs used to regulate autophagy affect the regulation of these genes. Finally, we offer valuable insights into specific mechanisms that should be targeted with appropriate drugs to leverage autophagy for the treatment of MM disease.

The mechanisms of autophagy

Autophagy is a recycling activity that is employed to maintain the survival of other cells and to maintain body homeostasis in case of stress such as nutrient deficiency and reactive oxygen compounds (ROS)[14]. In tumorigenesis, autophagy can work in a bidirectional manner by increasing disease progression or causing cancer cell death[15]. In addition, differentiations in autophagic balance might lead to drug resistance in cancer cells. Commonly autophagy-related genes are under-expressed or contain deletions in cancer cells[16]. In recent years, it has been declared that the loss of PTEN, a tumor suppressor, leads to a decrease in autophagy and thus plays a key role in shaping the fate of tumor cells[17]. The formation of autophagosomes primarily serves as a recycling process, not exclusively aimed at cancer cells. However, it works in tandem with the immune system in its specific function related to cancer[18]. Many autophagy-related proteins (ATG) are involved in the regulation of autophagy. The mechanisms of autophagy can be summarized into five items[19], as shown in Figure 1.

The initiation of phagophore formation is determined by the interaction between Beclin1 and Bcl-2, which is triggered by the phosphorylation of Bcl-2 by JNK-1. This results in the dissociation of Bcl-2 from Beclin1, which allows for the initiation of autophagy by the Beclin1/Vps34 complex. Recently, the role of Beclin1 and UV radiation resistance-associated gene (UVRAG) as a tumor suppressor and autophagy trigger in cancer was emphasized[20]. mTOR plays a pivotal role in regulating autophagy by inhibiting ATG1/ULK1-2 from

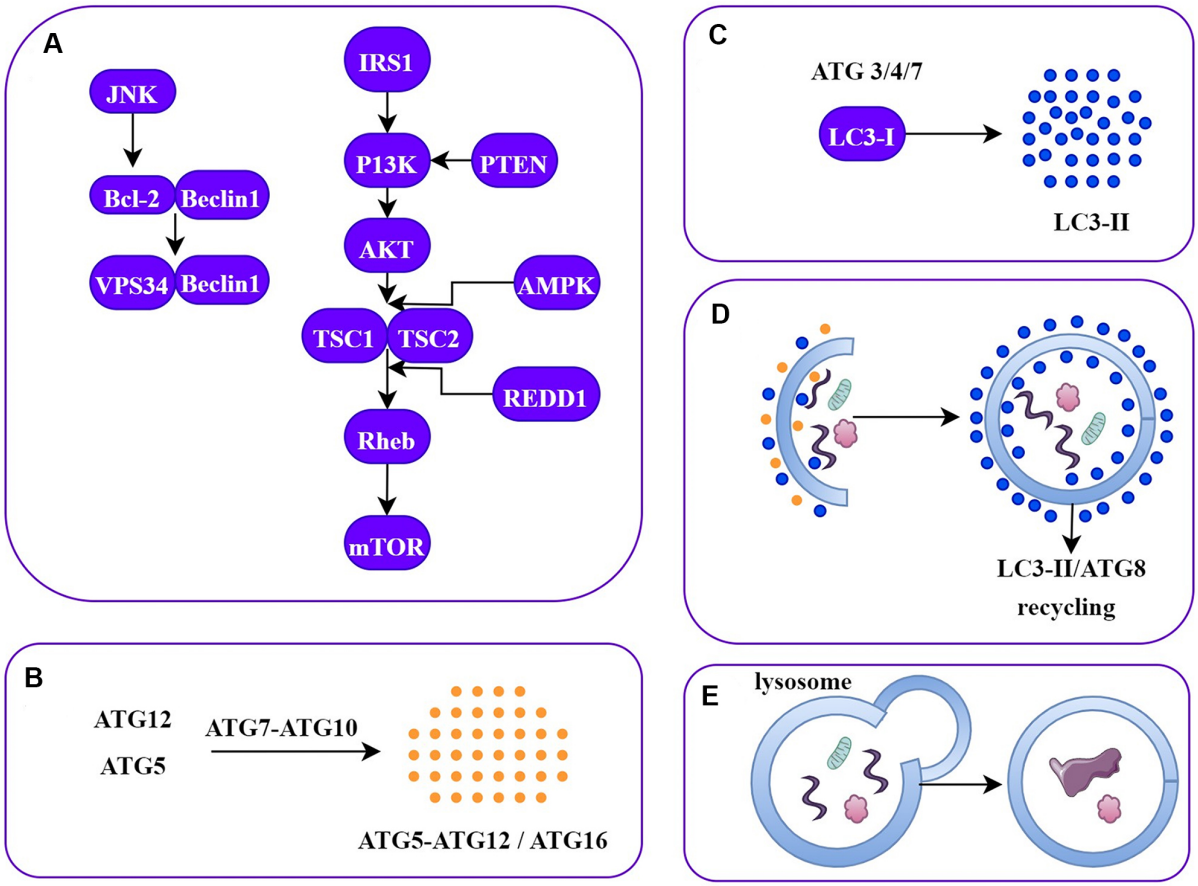

Figure 1. Schematic of the stages of autophagy. (A) Phagophore formation triggered by Beclin-1/VPS34 in response to a stress signal; (B) Multimerization resulting from ATG5/ATG12 conjugation and interaction with ATG16; (C) Embedding in the phagophore membrane by LC3 engraving; (D) Autophagosome formation by ATG4-directed LC3-II/ATG8 recycling; (E) Fragmentation of molecules following association with lysosome. ATG: Autophagy-related proteins.

the early stages. mTOR interacts with various proteins to form mTORC1/2 complexes, which activate different transduction pathways. Essentially, mTOR serves as the linchpin governing the balance between cell growth and the induction of autophagy. mTOR is inhibited by REDD1 and AMPK through Rheb under conditions of hypoxia or low ATP [Figure 1]. In addition, mTOR is activated via the IRS1, PI3K/AKT, TSC1/TSC2, and Rheb signaling pathways. Although three variants of autophagy have been proposed so far [microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy], degradation by lysosomal fusion is inevitable. Macroautophagy is characterized by the formation of an autophagosome, which consists of lipid bilayers so that large proteins and organelles can be broken down and recycled by the lysosome[19]. In microautophagy, the cell membrane spontaneously folds inwards to form a tube. The end of the tube buds and connects with the lysosome through vesicles[16]. Unlike others, CMA is the selective degradation of misfolded proteins by lysosomes via chaperones. The KFERQ motif in misfolded proteins is recognized by Hsc70 and forms a complex[21]. This complex is pulled in and destroyed by LAMP-2A in the lysosome membrane without the need for vesicles. Finally, they are degraded into the fundamental units by lytic enzymes (protease, lipase, and hydrolase) in the lysosome and are released to the cytoplasm for synthesizing new complex molecules.

Autophagy is a crucial factor in the circumstance of the disease, as MM cells are plasma cells that synthesize a large amount of immunoglobulin. Therefore, it is important to assess how autophagy functions, considering its dual impact as both a suppressor and supporter of the tumor[15]. ATG5, an important autophagy-related indicator, is cleaved upon exposure to Bortezomib and initiates autophagy in MM cells[22]. It is known that inhibiting autophagy in conjunction with proteasome inhibitors, which are effective in treating MM, presents a promising therapeutic avenue. Therefore, it is necessary to elucidate the fundamental mechanisms of autophagy at the forefront of MM research. In this review, autophagy mechanisms and autophagy modulators in MM disease were comparatively reviewed. This review emphasizes the significance of incorporating autophagy alongside appropriate medications and timing as a strategy to significantly extend the lifespan of MM patients.

AUTOPHAGY-RELATED MECHANISMS IN MM

MM cells use multiple mechanisms to evade cell death, including proteasome, unfolded protein response (UPR), and autophagy. MM cells that can escape from death develop resistance to drugs, which makes available therapies useless. The viability of MM cells is based on the proteasome-mediated degradation of misfolded proteins and non-functional newly synthesized proteins[23]. Therefore, autophagy can maintain the continuity of MM cells by averting the toxic stack of misfolded proteins. A basal level of autophagy is essential for the life cycle of MM cells. When these mechanisms collapse, apoptosis is finally induced by immune cells for the integrity and health of the organism[18]. Autophagy may be involved in the progression of MM from malignant plasma cells. Exemplarily, the enhancement of autophagy in plasma cells of MGUS and MM patients was only in the direction of MM[24]. A study conducted with biopsies of 89 patients with MM reported that patients with Beclin1 and LC3 biomarkers had a longer life expectancy[25]. It was revealed that elevated IFNγ in neutrophils isolated from MGUS and MM patients triggers JAK2/STAT3 signaling to form an autophagic survival system[26]. Recently, CD46, IKBKE, PARK2, ULK4, ATG5, and CDKN2A were considered in a three-cohort meta-analysis study that investigated the contribution of polymorphisms in autophagy-related genes to MM disease risk[27]. Considering all these, autophagy is related not only to MM progression or regression but also to MM development at the beginning. We can examine the mechanisms thanks to the fact that autophagy is effective in MM under the headings of UPR, bone marrow microenvironment (BMME), drug resistance, hypoxia, DNA repair and transcriptional regulation, and apoptosis.

UPR

The folding of proteins takes place in the endoplasmic reticulum (ER). Misfolded proteins are ubiquitinated by a system including ER chaperones and are degraded by the proteasome[28]. Besides contributing to the degradation of p62/SQSTM1 aggregated proteins via proteasomes, it is known that p62 can sustain cell survival by inducing autophagy[29]. Additionally, recent studies have shown that SQSTM1/p62 plays a crucial role in supporting proteostasis and is not just involved in working with the proteasome to degrade Ub-protein aggregates[30,31]. SQSTM1/p62 is a vital component of the autophagic reserve that helps the cell adapt to proteasome stress induced by proteasome inhibitors with the modification of the interactome[30]. In this sense, it can be interpreted that p62 functions in the regulation of autophagy. Autophagy plays a significant part in the transformation of B cells into plasma cells, and ATG5 mediates these tasks[32]. Mice with $Atg5^{f/f}$ were observed to have a higher ER stress with increased immunoglobulin synthesis compared to wild type[33]. Therefore, autophagy plays a role in guiding plasma cells to the bone marrow for adaptive immune responses. Myeloma cells are under constant ER stress due to the high content of immunoglobulins they synthesize and can easily induce the UPR[34]. Inhibition of the proteasome with chemotherapeutics makes the damaged proteins roll up, turn toxic, and trigger UPR[35]. UPR restores homeostasis and performs in conjunction with autophagy. Thus, autophagy is upregulated and initiates a survival mechanism to eliminate UPR.

The UPR proteolytic system is controlled by the signaling mechanism of IRE1/XBP-1, PERK/ATF4, and ATF6/cleaved ATF6. IRE1, PERK, and ATF6 are separated from GRP78, to which they are attached when UPR is triggered, which accounts for a signal transduction cascade[36]. The PERK signaling pathway conduces to the UPR system by suppressing protein synthesis. IRE1 participates both by augmentation of misfolded protein impairment and by enhancement of protein folding together with ATF6. The function of XBP-1 is of great importance in the autophagy mechanism in UPR and MM cells. IRE1 fulfills the cell viability role of autophagy by regulating itself and XBP-1 at the mRNA level[37]. Directing the maturation of plasma cells, XBP-1 is involved in autophagy through ATG5[38]. However, XBP-1 facilitates IRE1 binding to the BiP chaperone to downregulate the UPR function in B cells[39]. IRE1a/XBP-1 balance is critical in MM cells as it reduces ER stress[40]. Furthermore, it was reported that inhibition of XBP-1 splicing by IRE1 may be a treatment modality for MM cells[41]. Additionally, it was demonstrated that there is a correlation between elevated spliced XBP-1 levels and Bortezomib resistance[35]. XBP-1 is also involved in the regulation of ATG5 and Beclin1 via eIF2AK3 for the activation of autophagy[42]. Moreover, XBP-1 functions as a transcription factor in the signaling cascade between IRE1 and ATF6 cleavage in UPR[43]. XBP-1 influences the expression of genes grave in B cell differentiation, such as BAFF, APRIL, BCMA, and TACI[44]. In addition, these genes also affect NF-κB expression. In MM cells, the NF-κB pathway leads to the expression of cytokines, cell adhesion molecules, and cell growth and survival factors. NF-κB, which also mediates XBP-1 expression, contributes to the induction of UPR and autophagy in cancer cells[45]. It was previously mentioned that Beclin1 and LC3 work together in autophagosome formation. NF-κB raises the possibility of autophagy by augmenting Beclin1 expression[46]. PERK obstructs the phosphorylation of eIF2B, which leads to a significant reduction in the production of ATF4 and protein synthesis[47]. This mechanism serves as a crucial intervention point for regulating protein synthesis in cells. PERK can operate to diminish the oxidative stress response that may be motivated by unfolded proteins by activating NRF2[48]. In this sense, it plays a binary part in up or down-regulating UPR in autophagy. PERK labors with ATF4/6 to activate the transcription factor CHOP and to reduce ER stress[49]. From this point of view, PERK primarily functions to sustain cell life, but it can also operate cell death mechanisms in response to the strong signals it receives. In an experiment study where IRE1, ATF6, and PERK were individually knocked out using RNAi, it was indicated that PERK functions as a sensor of autophagy in MM cells[50]. An inhibitor that targets the PERK pathway via eIF2AK3 in MM cells holds great potential in inducing cell death and providing effective therapy[51]. This approach must be further explored to unlock its full potential in treating MM.

BMME

The BMME consists of cells and supporting elements. BMME stimulates a reputable part in the differentiation of B cells and their transformation into cancer[52]. As a result of the interaction of MM cells with stromal cells, IL-6, VEGF, and IGF-1 are secreted, and signaling pathways that cause tumor aggression, such as PI3K/AKT/mTOR, MEK/ERK, JAK/STAT3, and NF-κB, are activated [Figure 2]. Intercellular interactions between stromal cells and MM cells in the bone marrow and relationships with extracellular matrix (ECM) elements determine the fate of MM cells. Furthermore, adhesion molecules such as TNF-α, ICAM-1, LFA-1, and VLA-4 generated by stromal cells are responsible for the interaction of MM cells and bone marrow stromal cells (BMSCs). Stimulation of adhesion molecules for expression is mediated by NF-κB[53] and ultimately contributes to the development of drug resistance in MM cells. As another factor, IL-6 is an interleukin released from stromal cells and contributes to the clinical prognosis, apoptosis, drug resistance, and metastasis processes of MM[54]. Elevated IL-6 levels induce XBP-1 generation, which results in the enhancement of UPR and autophagy[24]. APRIL and BAFF ligands, which bind to BCMA secreted from B cells, promote XBP-1-mediated autophagy via NF-κB, assisting in the survival of MM cells[55]. It is argued that there may be crucial mechanisms for cancer-associated fibroblasts (CAFs) in autophagy in cancer cells[56]. CAFs from Bortezomib-resistant patients were observed to protect MM cells from apoptosis in the co-culture[56]. Because autophagy is induced by the inhibition of TGF-β in Bortezomib-resistant CAFs,

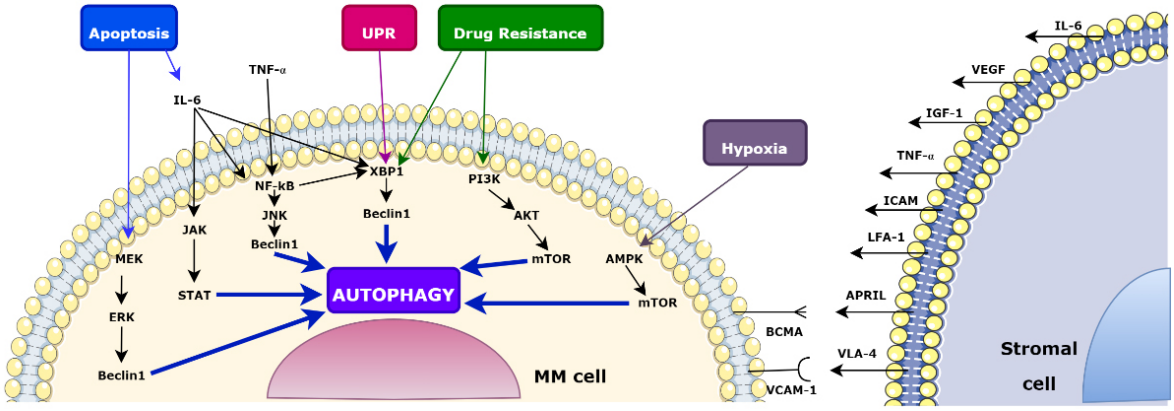

Figure 2. Causes and consequences of autophagy in MM cells within BMME. In the bone marrow, stromal cells secrete IL-6, VEGF, and IGF-1 due to intercellular interactions with MM cells. IL-6 stimulates UPR and autophagy through XBP-1. Apoptosis triggers autophagy via the JAK/STAT3 and MAPK/ERK pathways or IL-6. Autophagy and survival are promoted by the interaction of BCMA and APRIL through NF-κB and XBP-1. Adhesion molecules such as TNF-α, ICAM-1, LFA-1, and VLA-4 develop drug resistance by inducing autophagy via NF-κB. PI3K/AKT/mTOR inhibition promotes autophagy in drug-resistant cells. UPR upregulates HIF1-α through XBP-1 processing, which triggers autophagy through AMPK and mTOR. BMME: Bone marrow microenvironment; MM: multiple myeloma; UPR: unfolded protein response.

apoptosis is prevented by reducing ER stress[57]. Moreover, it was stated that mature adipocytes in BMME contribute to the induction of autophagy by MM cells to avoid apoptosis and to the development of drug-resistant phenotypes[58]. Autophagy also supports the immune system by taking part in the phagocytosis of microbes. LC3, IFN1, MHC class 2, and IL-6 aid in this process[5]. Apart from these, cancerous cells necessitate a higher nicotinamide adenine dinucleotide (NAD+) cycle and rely on the Nampt enzyme for regulation. The Nampt inhibitor FK866 suppresses the protective effects of BMME and demonstrates its effect in both resistant MM cell lines and MM patients[59]. Moreover, FK866 induces autophagy in MM through inhibition of mTORC1 and ERK1/2[59]. The involvement of the WNT signaling pathway in the self-renewal abilities and metastasis processes of MM cells has also been proven in another study[60]. Furthermore, it was found that exposure of MM cells to UV triggers the WNT pathway and induced autophagy[61].

Angiogenesis is an important element in the transition of the disease from MGUS to MM[62]. Although it is a distinguishing fact that angiogenesis occurs only in MM, various cytokines play a significant role in the process[63]. Pro-angiogenic cytokines are released by interactions among MM cells, stromal cells, and endothelial cells. Studies reported that MM cells secrete higher levels of bFGF, HGF, and VEGF than plasma cells, which promotes angiogenesis[64]. In a recent study, it was concluded that endothelial cells from patients with MM expressed higher levels of EGFR and its ligand HB-EGF than those from MGUS patients[65]. When EGFR is activated, it promotes blood vessel growth and assists endothelial cells in surviving. Blockade of HB-EGF-EGFR signaling limited the angiogenic potential of bone marrow endothelial cells and hampered tumor growth in an MM mouse model[65]. These findings suggest that HB-EGF-EGFR signaling could be a potential target in anti-angiogenic therapy for MM. Another study aimed to investigate the effect of combining Bortezomib and Hydroxychloroquine on plasma and endothelial cells isolated from patients with MM and MGUS[66]. The results showed that the combined treatment had different effects on MM cells and endothelial cells[66]. LC3B and p62 expressions induced autophagy in MM cells[66], and it was suggested that Bortezomib should be used in combination with an anti-angiogenic drug to enhance its effectiveness. Considering the dual role of autophagy in MM, it initially acts as a tumor suppressor, but may also promote tumor growth, survival, and therapy resistance. The relationship between EGFR and autophagy is intricate. In fact, inhibiting EGFR could trigger autophagy as a survival mechanism. Thus, by gaining a deeper understanding of the connection among EGFR, autophagy, and bone marrow angiogenesis, we can

potentially develop novel treatments for MM.

Drug resistance

Drug resistance is one of the major issues in MM and causes relapse and refractory of the disease. MM cells can avoid the toxic effects of chemotherapeutics via autophagy and thus develop drug resistance[14]. Bortezomib, which is frequently used in the treatment of MM and ratified by the FDA, triggers autophagy via UPR by inhibiting proteasomes[67]. Bortezomib resistance occurs frequently in MM, and different approaches are sought for treatment. Caspase-10 endorses the survival and resistance of MM cells by regulating autophagy[15]. Through induction of IRF4 in MM, caspase-10 cleaves BCLAF1, which competes with Beclin-1 for binding to Bcl-2 and inhibits autophagy[68]. Therefore, the sensitivity of MM cells to Bortezomib can be restored by developing caspase-10 inhibitors. The association of Bortezomib resistance with P-gp and drug resistance-related proteins in MM cells was demonstrated[69]. The increase in ATF4 expression after Bortezomib treatment in breast cancer cells[70] suggests that Bortezomib resistance can be overcome by combined suppression of autophagy and proteasomes. In a recent study on Bortezomib-resistant MM patients and cell lines, it was mentioned that upregulation of CMA may be a mechanism leading to resistance formation and targeting this mechanism could be a therapeutic target[71]. It was revealed that the CMA pathway was induced due to the ER stress caused by Bortezomib, and the expression of LAMP2A, which is an indispensable part of this pathway, was boosted[71]. However, it was stated that inhibition of the CMA pathway restored Bortezomib sensitivity, and the dual combination was more cytotoxic[71]. It was revealed that the Profilin-1 gene contributes to Bortezomib resistance in MM by boosting autophagy through Beclin1[72]. The combination of anti-β2M monoclonal antibodies with Bortezomib in MM cells promoted cellular death even in resistant cells by inhibition of autophagy[73]. Bortezomib response decreased in MM cells as a result of suppression of NEDD4L, which induced autophagy by binding the 19S proteasome[74]. A mechanism for the NEK2/USP7/Beclin-1 complex was identified in autophagy-induced Bortezomib resistance in MM patients[75]. As a result of Bortezomib treatment applied to MM cells, autophagy was incited by GRP78 in cells[76]. Furthermore, it was displayed that HSPA5 expression was upregulated in Bortezomib-resistant cells[76]. It was declared that transfection of mir145-3p into MM cells suppresses HDAC4 and mTORC1 and causes sensitivity to Bortezomib due to autophagy[77].

Autophagy functions in the development of resistance to many chemotherapeutics used in the treatment of MM. In a related study, by using primary patient samples and Dexamethasone-resistant MM cell lines, Beclin1 and LC3/ATG8 expressions were found to be inversely proportional to the p62 level[78]. In this case, the authors claimed that autophagy leads to drug resistance in MM, and Beclin1/p62 is a biomarker. Additionally, antiestrogen binding site ligands regulated the cholesterol mechanism via the PI3K/AKT/mTOR pathway, inducing autophagy and overcoming Dexamethasone resistance[79]. The relationship of Carfilzomib resistance with autophagy in MM cells and the contribution of the KLF4 transcription factor were obvious[80]. KLF4 attaches to the p62 gene promoter and upregulates its expression. It was emphasized that Doxorubicin-induced resistance in MM cells is mediated by DEPTOR, and autophagy plays a main role in this resistance[81].

Hypoxia

Tumor cells have a high metabolic rate and require large amounts of nutrients and oxygen. The needs of the rapidly growing tumor mass cannot be met at the same rate through the vessels, and a hypoxic environment develops. Cancer cells can continue to proliferate by inducing HIF1-α and adapting to these unfavorable conditions. HIF1-α triggers autophagy by inhibiting Bcl-2/Beclin1 and upregulating ATGs through BNIP3 and its ligand[82]. However, tumor cells can initiate autophagy independently of HIF1-α through the activation of mTOR or AMPK[83]. Since BMME has a low pO_2 level compared to blood, MM cells can easily

adapt to the hypoxia environment[84]. The hypoxia state around the MM cell provokes the induction of UPR and autophagy due to the aggregation of the unfolded protein[85] because many proteins need O_2 in order to be folded properly. Due to hypoxia-induced O_2 deficiency, sufficient ATP cannot be produced from glucose. In this case, MM cells can also induce starvation-induced autophagy[86]. It was reported that upregulation of Hexokinase-2 in hypoxia conditions intensifies autophagy in MM cells and introduces it as an antiapoptotic feature[87]. UPR initiates hypoxia by upregulating the expression of HIF1-α and VEGF via XBP-1[88]. In addition, activation of mTORC1, an autophagy-related factor, was found to increase susceptibility to hypoxia in MM cells[89].

DNA repair and transcriptional regulation

Dysfunctional DNA repair pathways facilitate the progression and metastasis of MM disease from its initial stages to the development of drug-resistant phenotypes, because autophagy can be induced through DNA repair mechanisms[90]. The activation of autophagy via the mTORC1/TSC2/ULK1 pathway is triggered by the ATM-mediated phosphorylation of AMPK[91,92]. Additionally, ATM enables the inhibition of mTOR through the activation of Che-1 via REDD1 and DEPTOR[93]. Furthermore, stress-induced Sestrin2 binds to GATOR2, thereby suppressing mTORC1[94]. NF-κB, also activated by ATM, leads to the activation of Beclin1 and induction of autophagy[95]. ROS-induced DNA damage assists in the suppression of AMPK and triggering of autophagy by activation of PARP1[96]. ATM can also induce autophagy through the regulation of p53. p53 induces the initiation of autophagy through the upregulation of PTEN and AMPK[97]. Moreover, DAPK regulated by p53 facilitates autophagy through its connection with Beclin1 phosphorylation and VPS34 activation[98].

Histone deacetylases (HDACs) are crucial transcription regulators and can control functional proteins in signaling pathways. HDAC6 is critical in MM due to its connection with UPR. Because of its deficiency, autophagy can be induced by forming aggresomes. Therefore, it is a promising target for patient treatment. HMGB1 is a DNA-binding protein that contributes to the functionality of nucleosomes and causes tumor progression. HMGB1 expression is increased during the treatment of various cancers with different chemotherapeutics[99]. HMGB1 is involved in autophagy and DNA damage repair, and the knockdown of HMGB1 in MM cells restores mTOR-mediated Dexamethasone sensitivity[100]. Additionally, it was reported that HMGB1 expression increased in Bortezomib-resistant MM cells, and the combination of Bortezomib and Lycorine reversed the resistance[101]. Furthermore, it was emphasized that MALAT1, a lncRNA, caused the upregulation of HMGB1 in MM cells, and the knockdown of MALAT1 caused a decrease in the expressions of Beclin1 and LC3B together with HMGB1 in MM[102]. In light of these findings, it is clear that targeting HMGB1 in drug-resistant MM patients is an essential and promising therapeutic approach.

Apoptosis

Apoptosis against cellular stress is the first type of cell death pathway and is used to eliminate situations where autophagy cannot be overcome. Depending on the stress state of the cell, these death mechanisms can block or trigger each other. Apoptosis in MM cells can be induced by IL-6, VEGF, IGF1, SDF1, and FGF factors via the JAK/STAT3 and MAPK/ERK pathways[16]. It was reported that drug-resistant MM cells induce apoptosis via the Apo2L/TRAIL pathway[103]. The expression of Mcl-1, a proliferation marker of MM cells, increased by JAK/STAT3 and VEGF, resulting in resistance to apoptosis[104]. The importance of the Bcl-2/Bax ratio in MM apoptosis is indisputable[105], and the increase in Bcl-XL protein leads to the inhibition of apoptosis via IL-6. NF-κb's involvement in MM cell apoptosis, metastasis, and drug resistance was investigated[106]. Autophagy helps to alleviate metabolic stress, but when apoptosis and autophagy are both impaired, it can lead to necrotic cell death[13]. Therefore, autophagy plays a role in suppressing tumors by reducing metabolic stress and preventing necrotic cell death, along with apoptosis[13].

The similarity of proteins in the apoptosis and autophagy pathways is a well-known phenomenon in MM, which highlights the complex interplay between these cellular processes[18]. As an example, Beclin1 executes its function as a tumor suppressor through regulation by AMBRA1, Bif1, and UVRAG. Both siRNA-mediated inhibition of Beclin-1 and autophagy inhibition by 3-methyladenine and Chloroquine resulted in apoptosis of MM cells[107]. Furthermore, inhibition of autophagy with 3-methyladenine augmented Oridonin-dependent apoptosis by regulating ROS and SIRT1 in MM cells[108]. Beclin1 inhibition extinguishes both autophagy and apoptosis, because Bcl-2 inhibits apoptosis by MOMP inhibition and stops autophagy by tethering Beclin1 or AMBRA1[109]. It was claimed that HMGB1 disturbs the equilibration between Beclin1 and Bcl-2 in the resistance to Bortezomib in MM[101]. AMBRA1 induces autophagy and suppresses apoptosis[110]. UVRAG induces autophagy by activating Beclin1, while Bif1 induces tumor suppression via autophagy[111]. In addition, a high expression of p38/MAPK engenders autophagy, while a low expression causes apoptosis[112]. STAT3 regulates many autophagy-related genes, such as FOXO1/3, by sequestering eIF2AK2 to fulfill this function[113]. It was suggested that the nutritional status marker mTORC1 could be responsible for drug resistance in MM via the AKT/ERK signaling pathway[114]. In a study with MM patients and cell lines, it appeared that gene expressions related to the PI3K/AKT/mTOR pathway and autophagy were higher in resistant groups[115]. Additionally, it was also unveiled that inhibition of PI3K/AKT/mTOR signaling promotes autophagy and apoptosis[115]. Autophagy can also induce apoptosis by aggregation of caspase 8[116]. Although p53 is an important biomarker in cancer, its role in the control of cell cycle and apoptosis in MM was clarified[105].

Recent studies reveal that MM cells can reshape cellular traffic mechanisms to combat ER stress. FAM46C, which is mutated in more than 10% of MM patients, is frequently emphasized. In a study on MM cells, it was reported that reactivation of FAM46C triggered apoptosis and ER stress by forming a complex with FNDC3A, an ER-associated protein[117]. The FAM46C and FNDC3A complex increases the exocytosis of lysosomes, which causes changes in cellular traffic and secretion[117]. This leads to aggregate accumulation and negative regulation of autophagy in MM cells[117]. Eventually, MM cell-specific FAM46C/FNDC3A-mediated tumor suppression occurs by induction of apoptosis[117]. Another study highlights the tumor suppressor and therapeutic role of FAM46C[118]. FAM46 interaction with FNDC3 proteins stabilizes ER-targeted protein mRNAs and increases ER growth and Ig secretion[118]. However, when the proteasomal balance is disrupted, p62 associates with FAM46C and prevents its interaction with FNDC3 proteins[118]. The p62/FAM46C/FNDC3 ensures the survival of MM cells, and targeting this pathway with proteasome inhibitors appears as a mechanism that enables selective cell death.

THERAPEUTICS FOR AUTOPHAGY IN MM

Autophagy plays a crucial role in the development of MM, a disease where plasma cells produce large quantities of immunoglobulin. Combining autophagy inhibition with proteasome inhibitors is an effective MM treatment. Since autophagy can work in both directions, strategies to induce or inhibit autophagy could be helpful in the treatment of MM. Benefits can be obtained by inhibiting the tumor mass-preserving effect of autophagy or by directly triggering autophagy in MM cells. Therefore, suppressing the PI3K/AKT/mTOR signaling pathway to induce autophagy in MM cells may be a therapeutic option. The PI3K/AKT/mTOR signaling pathway is associated with p38/MAPK, PTEN, p53, SIRT1, IGF-1 and ROS. Since the MAPK/ERK signaling pathway is in connection with mTOR, its suppression in MM may inhibit autophagy. JNK may have a tumor suppressor task due to its capacity to regulate the function of Beclin1. Ca^{2}+ itself, as a signaling molecule, can trigger autophagy through its ability to activate Beclin1 and AMPK. The above-mentioned signaling pathways can be regulated with drugs to achieve the desired autophagy benefit in MM. For this reason, Table 1 lists drugs that can inhibit or induce autophagy by various mechanisms, especially in MM. Table 2 provides an overview of the most promising autophagy modulators clinically tested in MM patients.

Table 1. Autophagy modulators and functioning mechanisms in MM

Mechanism	Drug	Effect	Ref.
AMPK activators	Metformin	Activation	[119-121]
	Spermidine		[122]
AMPK and mTOR inhibitors	Resveratrol	Activation	[123,124]
Autophagy flux inhibitors	Elaiophylin	Inhibition	[125]
	4-Acetylantroquinonol B		[126]
Autophagy inducer	Tat-Beclin1 peptide	Activation	[127]
BTK inhibitors	Ibrutinib	Activation	[128]
Ca^2-ATPase inhibitors	Thapsigargin	Inhibition	[129,130]
Class I PI3K inhibitors	CH5132799	Activation	[131]
	GDC-0941		[132]
Class III PI3K inhibitors	3-Methyladenine	Inhibition	[107,108,133,134]
	Wortmannin		[135]
	LY294002		[136]
HDAC inhibitors	Vorinostat	Activation	[137,138]
Lysosomal alkalizers	Chloroquine	Inhibition	[107,139,140]
	Hydroxychloroquine		[66,134]
	Lys05		[141]
Lysosome membrane permeabilization	Thymoquinone	Inhibition	[142]
mTORC1/2 inhibitors	Rapamycin	Inhibition	[124,134]
	Everolimus		[143,144]
	Deforolimus		[145]
	Temsirolimus		[146]
	AZD8055	Activation	[147]
	Torin1		[148]
	PP242		[149]
PI3K/AKT inhibitors	Perifosine	Activation	[150]
PI3K/mTOR inhibitors	Apitolisib	Activation	[151]
Protease inhibitor	Pepstatin A	Inhibition	[152]
	E64d		[68,153]
Vacuolar H-ATPase inhibitors	Bafilomycin A1	Inhibition	[140,154]
	Concanamycin A		[155]
VPS34 kinase inhibitors	Paclitaxel	Inhibition	[156,157]

MM: Multiple myeloma.

Lysosomal alkalizers

Lysosomes are important organelles of autophagy, and changes in pH levels prevent the activity of lysosomal enzymes and the fusion of autophagosomes with lysosomes. As mentioned before, the life of MM cells is dependent on the protein synthesis cycle. Therefore, the combination of lysosomal alkalizers and proteasome inhibitors or alkylating agents is a powerful therapeutic option for the selective killing of MM cells. It is known that Hydroxychloroquine, which is widely used in the treatment of malaria, can inhibit autophagy in cancer cells[162]. The combination of Bortezomib and Hydroxychloroquine increased cellular cytotoxicity via autophagy in MM patient samples[66]. In the Phase 1 study of MM patients, the same treatment combination not only improved disease symptoms but also reduced side effects[158]. In Phase II trials, whose Chloroquine was administered in combination with Bortezomib and Cyclophosphamide, a curative effect was shown for relapsed and refractory MM patients[139].

Table 2. Summary of autophagy therapeutics in MM clinical trial

Title	Therapeutics	Phase	Publication	Identifier
A pilot study of infusional Cyclophosphamide and pulse Dexamethasone with Rapamycin or Hydroxychloroquine in patients with relapsed or refractory multiple myeloma	Hydroxychloroquine Rapamycin	Early phase 1	-	NCT01396200
A Phase I/II trial of Hydroxychloroquine added to Bortezomib for relapsed/refractory myeloma	Bortezomib Hydroxychloroquine	Phase 1	[158]	NCT00568880
A Phase I dose escalation study of Hydroxychloroquine with infusional Cyclophosphamide, pulse Dexamethasone, and Rapamycin in patients with relapsed or refractory multiple myeloma	Cyclophosphamide Dexamethasone Hydroxychloroquine Sirolimus	Phase 1	-	NCT01689987
A Phase II, trial of Chloroquine in combination with VELCADE and Cyclophosphamide in patients with relapsed and refractory myeloma	Velcade Cyclophosphamide Chloroquine	Phase 2	[139]	NCT01438177
A Phase 1B/2 multicenter, open label, dose-escalation study to determine the maximum tolerated dose, safety, and efficacy of ACY-1215 (RICOLINOSTAT) in combination with Pomalidomide and low-dose Dexamethasone in patients with relapsed and refractory multiple myeloma	Ricolinostat Pomalidomide Dexamethasone	Phase 1 Phase 2	-	NCT01997840
A Phase 1/2, open-label, multicenter study of ACY-1215 administered orally as monotherapy and in combination with Bortezomib and Dexamethasone for the treatment of relapsed or relapsed/refractory multiple myeloma	Ricolinostat	Phase 1 Phase 2	[159]	NCT01323751
A Phase 1/2, open-label, multicenter study of ACY-1215 (Ricolinostat) in combination with Lenalidomide and Dexamethasone for the treatment of relapsed or relapsed/refractory multiple myeloma	Ricolinostat Lenalidomide Dexamethasone	Phase 1	[160]	NCT01583283
An open-label phase I study of the safety and efficacy of RAD001 in combination with Lenalidomide in the treatment of subjects with relapsed and relapsed/refractory multiple myeloma	Everolimus Lenalidomide	Phase 1	[161]	NCT00729638
Phase II trial of RAD001 in relapsed/refractory multiple myeloma	Everolimus	Phase 2	-	NCT00618345
An open-label phase 1 study of Metformin and Nelfinavir in combination with Bortezomib in patients with relapsed and/or refractory multiple myeloma	Bortezomib Metformin Nelfinavir	Phase 1	-	NCT03829020
Phase II trial, open label, clinical activity of Metformin in combination with high-dose of Dexamethasone (HDdexa) in patients with relapsed/refractory multiple myeloma	Metformin Dexamethasone	Phase 2	[120]	NCT02967276

MM: Multiple myeloma.

HDAC inhibitors

The performance of the HDAC6 inhibitor Panobinostat added to the MM treatment protocol in Phase 3 trials is clear[163] because HDAC6 inhibitors work together with UPR in toxic protein aggregation to promote autophagy. Additionally, HATs and HDACs can control the acetylation status of autophagy-related genes. An example of HDAC inhibitors inducing autophagy is the inactivation of mTOR by Vorinostat. Autophagy is suppressed by phosphorylating ULK1 through mTOR. The HDAC inhibitor Vorinostat triggers autophagy in tumor cells through the production of ROS[164]. The combination of Suberoylanilide hydroxamic acid (Vorinostat), 17-allylamino-17-demethoxy-geldanamycin (Tanespimycin), and Clonazepam overcame the complication of peripheral neuropathy by inducing autophagy via HSP70 or LAMP-2A[165]. Moreover, the efficacy of the combination of Clarithromycin, a high-spectrum antibiotic, in MM and various cancers has been previously reported[166]. Clarithromycin's suppression of autophagy by reducing cytokines such as IL-6, which is an important factor in MM development and drug resistance, makes it a possible candidate. It was shown that the combination of Bortezomib, Lenalidomide, and Clarithromycin would also be suitable for therapy without Dexamethasone in MM patients with diabetes[167]. The combination of Vorinostat, Bortezomib, and Clarithromycin in MM cells causes cell death and upregulation of CHOP genes that stimulate ER stress[137]. In this sense, combinations of proteasome inhibitors and next-generation HDAC6 inhibitors may serve as therapeutic options to increase autophagy in MM cells.

Vacuolar H-ATPase inhibitors

Vacuolar type H-ATPases are pumps responsible for regulating intracellular organelle pH. Inhibiting these pumps with specific inhibitors blocks autophagy and prevents cell metastasis. The combined effect of Bortezomib and Bafilomycin A1 in MM cells is more cytotoxic than their use alone, which reveals the link between UPR, autophagy, and ER stress[154]. Furthermore, a combination of Ixazomib and autophagy inhibitors Bafilomycin A and Chloroquine increases cytotoxicity and cell death via JNK[140]. Therefore, the combination of vacuolar-type H-ATPase inhibitors with proteasome inhibitors stands as a potential therapeutic option. Additionally, inhibition of autophagic flux is also a treatment option. In a recent study, the treatment of p53 mutant MM cells with Elaiophylin suppressed autophagy and resulted in cell death by apoptosis[125].

AMPK activators and mTOR inhibitors

AMPK plays a critical role in responding to stress caused by metabolic disorders. It helps in maintaining the balance between anabolic and catabolic reactions, which is crucial for the stability of the cell and energy preservation. In cancer treatment, AMPK activators are used to trigger autophagy and cell death. In peripheral neuropathy, one of the best-known side effects of Bortezomib treatment in patients, pathological changes occur in Schwann cells. Metformin, which is used as an antidiabetic drug, in combination with Bortezomib, suppressed GRP78 and promoted autophagy[120]. Furthermore, Metformin was recently shown to induce apoptosis and necrosis in MM cells and mouse models[119]. Additionally, Metformin applied to MM cell lines and mouse models promoted cell proliferation arrest and autophagy by AMPK and mTORC1/2 regulation[121]. Thus, AMPK activators are a promising option for inducing autophagy by selectively targeting MM cells.

mTOR plays a cornerstone role in autophagy by regulating proliferation and metabolism. Resveratrol induces cell cycle arrest, apoptosis, and autophagy. In a study conducted to prove this, it was emphasized that Resveratrol induces autophagy and apoptosis in MM cells by inhibiting AMPK and mTOR[123].

PI3K/AKT/mTOR inhibitors

The PI3K/AKT/mTOR signaling pathway promotes the survival and proliferation of tumor cells. Therefore, inhibition of one or more proteins on the signaling pathway causes tumor regression. A report on testing autophagy-regulating drugs in MM cells stated that Rapamycin can induce apoptosis and autophagy, Hydroxychloroquine can inhibit autophagy and induce apoptosis, and 3-methyladenine can only inhibit autophagy[134]. Furthermore, the combination of 3-methyladenine and VEGF inhibitor Bevacizumab in MM cells promoted apoptosis[133]. In a recent study, NVP-BEZ235 was discovered to induce autophagy through the mTOR2-Akt-FOXO3a-BNIP3 signaling pathway in MM cell lines, mouse models, and primary samples[168]. Targeting the PI3K/AKT/mTOR signaling pathway, which is central to cell life and death choices, is a good strategy and a powerful therapeutic option for clinicians.

Other drugs or compounds

Glycosphingolipids located outside the cell membrane are important in cell adhesion, cell-cell interactions, and oncogenesis. In MM and MGUS preclinical models, it was shown that Eliglustat can inhibit autophagy through the glycosphingolipid mechanism, thereby maintaining osteoclastogenesis and preventing bone loss[169]. The tumor burden-reducing effect of the combination of hexokinase-2 inhibitor 3-bromopyruvate and Bortezomib was published[87]. It was reported that Solamargine alone induces autophagy, and its combination with Bortezomib may be a good treatment strategy[170]. The induction of autophagy in MM cells by inhibitors of the isoprenoid biosynthesis pathway is a potential target for developing new therapeutic drugs[171].

Dihydroartemisinin induced autophagy and apoptosis via WNT/β-catenin and P38/MAPK in MM cell lines and mouse models[172]. Hsp90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin triggered autophagy over mTOR inhibition in MM cells[173]. Clioquinol engendered autophagy by causing mTOR downregulation in MM and leukemia cells[174].

Trifluoperazine, an antipsychotic drug, caused the inhibition of autophagy by NUPR1 overexpression in MM cells and led to the induction of apoptosis[175]. Betulinic acid performs its cytotoxicity in MM cells by inducing autophagy or apoptosis via the PP2A switch[176]. It was reported that Fingolimod regulates apoptosis by inducing autophagy in MM cells[177].

Drug discovery

Currently, there is no known treatment option to be considered definitive for targeting autophagy in cancer. This is because traditional cell cultures do not fully describe the complex interaction between cancer cells, other cells, and the microenvironment. However, 3D cell cultures are more effective in depicting the tumor microenvironment than traditional cultures, and they better reflect the differentiation and proliferation of tumor cells[178]. Microfluidic systems are biomedical platforms that enable the controlled distribution of small amounts of liquids, which facilitates the simultaneous execution of multiple tests[179]. These devices allow for more accurate and quicker studies of the tumor microenvironment, metastasis, and drug resistance[1]. They also enable drug screening to be carried out rapidly and precisely, which assists in identifying new drug candidates with more efficiency[180].

CONCLUSION

MM is a disease of plasma cells and is very sensitive to protein turnover processes. Autophagy, a mechanism linked to cell death, contributes to the progression of the disease by activating the UPR system in MM cells. Proteasome inhibitors, commonly employed in MM, induce UPR and ER stress. However, inhibiting proteasomes not only triggers autophagy but also leads to drug resistance. Therefore, there is a close interplay among UPR, autophagy, apoptosis, and drug resistance. The combination of proteasome inhibitors such as Bortezomib and Carfilzomib, which are frequently used in the treatment of MM, and autophagy inhibitors can effectively induce cell death. Additionally, agents that inhibit heat shock proteins also show therapeutic potential in MM by initiating autophagy. Furthermore, as in all cancer processes, autophagy in MM is affected by BMME, DNA repair, and transcriptional regulation, crucial aspects in the development of MM disease. The addition of autophagy inhibitors to agents that cause DNA damage will prevent MM cells from evading death. The widespread use of HDAC6 inhibitors in treating MM cells deactivates the UPR protection and induces autophagy. Clinically, PI3K/AKT/mTOR inhibitors prove effective by restricting MM cell growth, making cell death inevitable. Furthermore, even in normal cells, stress-causing starvation and hypoxia conditions trigger autophagy. Consequently, VEGF and EGFR inhibitors, which will prevent MM cells from feeding, and HIF1-α inhibitors, which will cause oxygen deprivation, become beneficial. Moreover, small molecules that target genes involved in the autophagy mechanism also have a therapeutic potential. There is a considerable volume of both completed and ongoing clinical trials exploring this avenue. However, a clinically promising drug has not yet been released, and there is no standardized autophagy treatment yet. In this regard, leveraging 3D and microfluidic systems holds the potential to develop disease models. These platforms have the capability to generate results more rapidly than traditional clinical trials. Additionally, reconstructing disease models with 3D models and microfluidic systems can lead to the identification of new autophagy modulators[1,178]. Moreover, it is possible to conduct tests on new agents before administering them to the patient and to develop appropriate strategies that can be tailored according to the patient's needs. This enables the utilization of autophagy, similar to apoptosis, in halting or even eradicating the progression of MM.

DECLARATIONS

Authors' contributions

Conceptualized, did the literature review, designed the review, and drafted the manuscript: Kozalak G
Edited the manuscript, supervised the process, and critically revised the article: Koşar A
Both authors read and agreed with the published version of the manuscript.

Availability of data and materials

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Conflicts of interest

Both authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

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Consent for publication

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Cancer Drug Resistance

Review

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Mechanisms of tyrosine kinase inhibitor resistance in renal cell carcinoma

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Abstract

Renal cell carcinoma (RCC), the most prevalent type of kidney cancer, is a significant cause of cancer morbidity and mortality worldwide. Antiangiogenic tyrosine kinase inhibitors (TKIs), in combination with immune checkpoint inhibitors (ICIs), are among the first-line treatment options for patients with advanced RCC. These therapies target the vascular endothelial growth factor receptor (VEGFR) tyrosine kinase pathway and other kinases crucial to cancer proliferation, survival, and metastasis. TKIs have yielded substantial improvements in progression-free survival (PFS) and overall survival (OS) for patients with advanced RCC. However, nearly all patients eventually progress on these drugs as resistance develops. This review provides an overview of TKI resistance in RCC and explores different mechanisms of resistance, including upregulation of alternative proangiogenic pathways, epithelial-mesenchymal transition (EMT), decreased intracellular drug concentrations due to efflux pumps and lysosomal sequestration, alterations in the tumor microenvironment including bone marrow-derived cells (BMDCs) and tumor-associated fibroblasts (TAFs), and genetic factors such as single nucleotide polymorphisms (SNPs). A comprehensive understanding of these mechanisms opens the door to the development of innovative therapeutic approaches that can effectively overcome TKI resistance, thereby improving outcomes for patients with advanced RCC.

Keywords: Antiangiogenic tyrosine kinase inhibitors, renal cell carcinoma, acquired resistance, sunitinib, tumor microenvironment, immune checkpoint inhibitors

INTRODUCTION

Kidney cancer is the 6th most common cancer in men and the 9th most common cancer in women in the United States, and there will be an estimated 82,000 new cases and 15,000 deaths in 2023[1]. Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for over 90%-95% of all cases[2]. With an annual incidence rate of 17.1 per 100,000 individuals and a mortality rate of 3.6%, RCC has become a significant public health concern, resulting in substantial morbidity and mortality[3]. RCC originates from renal tubular epithelial cells and comprises a diverse and heterogeneous group of pathologies[4]. RCC is categorized into multiple subtypes, the most common of which is clear cell RCC (ccRCC), accounting for approximately 75% of all cases and 85% of metastatic cases[5,6]. Other subtypes include papillary, chromophobe, collecting duct, and renal medullary carcinomas. Risk factors for RCC include age, male sex, tobacco use, hypertension, and genetic syndromes such as von Hippel-Lindau (VHL)[7,8].

Management of RCC varies based on disease stage and characteristics. For localized disease, treatment strategies encompass active surveillance, nephrectomy, radiofrequency ablation, and cryotherapy[9,10]. Adjuvant systemic therapies including antiangiogenic targeted therapies have thus far failed to demonstrate a benefit in overall survival, although there are several ongoing trials in this space[11]. The management of metastatic disease, predominantly ccRCC, is more complicated. Until the mid-2000s, systemic cytokines including interleukin-2 (IL-2) and interferon-alpha (IFN-α) were commonly used, but response rates to these therapies were poor[12]. The initial breakthrough in targeted therapy for RCC occurred in 2005 with the approval of sorafenib, a tyrosine kinase inhibitor (TKI) against vascular endothelial growth factor (VEGF). In 2009, bevacizumab, a monoclonal antibody against VEGF, was approved in combination with IFN-α. Subsequently, other multitargeted small molecule antiangiogenic TKIs emerged as treatment options and significantly improved progression-free survival (PFS) and overall survival (OS)[13]. Another class of agents, the mammalian target of rapamycin (mTOR) inhibitors, were introduced during the same period. In recent years, the addition of immune checkpoint inhibitors (ICIs) to TKIs in the frontline setting has reshaped the treatment landscape of advanced RCC[14].

TKIs are competitive inhibitors that bind to the kinase domain of their target receptor tyrosine kinase (RTK). RTKs are transmembrane proteins possessing an extracellular ligand-binding domain and an intracellular kinase domain, enabling them to transmit signals across the plasma membrane. Upon ligand binding, RTKs undergo dimerization and autophosphorylation, leading to kinase domain activation. This provides a docking site for proteins, enabling them to direct a cascade of intracellular events that regulate cellular proliferation, differentiation, survival, and migration [Figure 1][15]. Dysregulation of RTK signaling via constitutive autophosphorylation resulting in ligand-independent kinase activity has been implicated in many cancers, making RTKs attractive therapeutic targets[16]. In RCC, the signaling pathways initiated by overactive VEGF receptor (VEGFR) lead to enhanced endothelial cell migration, proliferation, permeability, survival, and lymphangiogenesis[17]. Antiangiogenic TKIs have been developed to mute this response by binding at or adjacent to the ATP-binding site, preventing autophosphorylation[18,19]. These drugs target various kinases, including VEGFR, platelet-derived growth factor receptor (PDGFR), c-Kit, FMS-related receptor tyrosine kinase 3 (FLT-3), and rearranged during transfection tyrosine-protein kinase (RET)[20]. The majority of TKIs are multitargeted and have activity against several kinases with varying potency. Importantly, all TKIs commonly used in the treatment of advanced RCC possess activity against VEGFR[21]. These include sunitinib, sorafenib, pazopanib, axitinib, cabozantinib, and lenvatinib.

Despite the success of these targeted agents, antiangiogenic TKI resistance is common and poses significant obstacles to achieving durable responses in the treatment of RCC. For instance, the majority of RCC patients who start on sunitinib will develop resistance within 6 months of treatment[22]. In this review, we

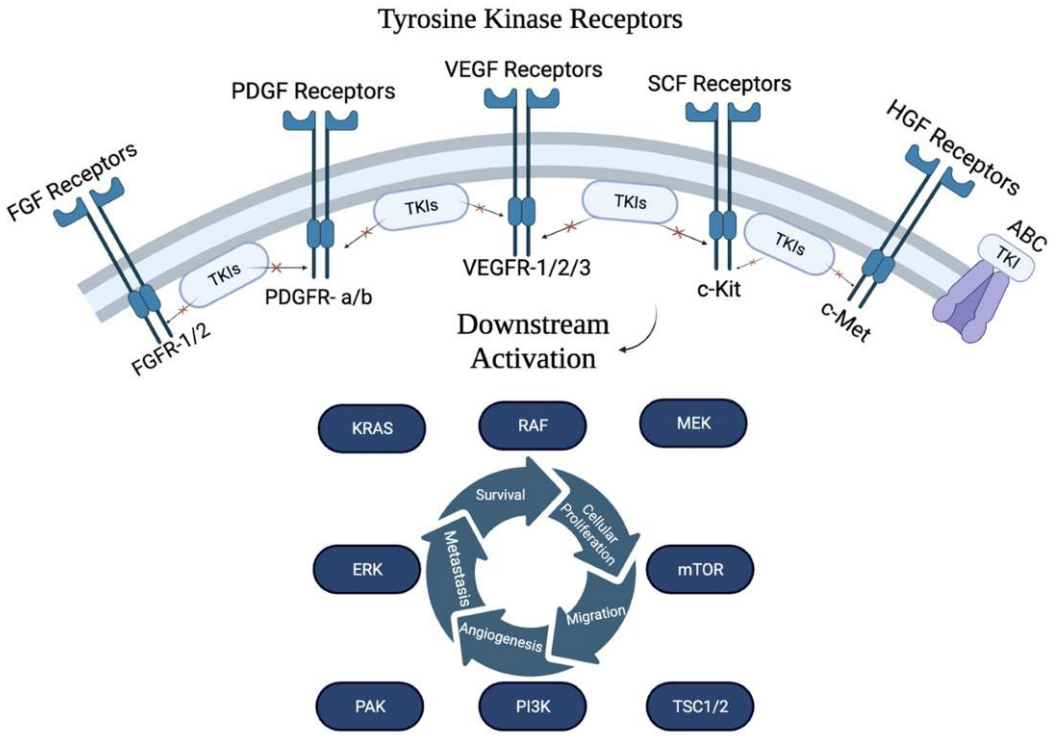

Figure 1. Common receptor tyrosine kinases and their downstream signaling targets. ABC: ATP-binding cassette; ERK: extracellular signal-related kinases; FGF: fibroblast growth factors; FGFR: FGF receptor; HGF: hepatocyte growth factor; mTOR: mammalian target of rapamycin; PDGFR: platelet-derived growth factor receptor; PI3K: phosphoinositide-3-kinase; TKIs: tyrosine kinase inhibitors; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

aim to explore the molecular mechanisms underlying TKI resistance in RCC and shed light on potential strategies to overcome this phenomenon. By elucidating the complexities of TKI resistance, we hope to pave the way for the development of novel therapeutic approaches that can improve outcomes for patients with advanced RCC.

RATIONALE FOR ANTIANGIOGENIC TKIS IN RCC

The treatment of RCC stems from the underlying molecular and genetic alterations that drive tumorigenesis and progression. Almost all hereditary and most sporadic cases of RCC involve mutations leading to loss of tumor suppressor gene function. The most common mutation in ccRCC is the loss of the VHL via loss of chromosome 3p, seen in over 90% of sporadic cases[23]. This leads to the loss or impaired function of VHL tumor suppressor protein (pVHL). Normally, pVHL targets the alpha subunits of hypoxia-inducible factor (HIF) for proteasomal degradation through prolyl hydroxylases. In the absence of pVHL, HIF, particularly HIF-2α, accumulates and acts as a transcription factor to trigger the overexpression of hypoxia-inducible genes such as VEGF, platelet-derived growth factor-BB (PDGF-BB), transforming growth factor-alpha (TGF-α), c-Met, cyclin D1, and CXCR4[24]. These genes play a crucial role in angiogenesis, cell proliferation, and tumor growth. HIF-2α also upregulates the production of c-Myc, a transcription factor implicated in the progression of various cancers[25]. Antiangiogenic TKIs disrupt this aberrant molecular cascade by specifically targeting the dysregulated signaling pathways driven by HIF-2α and its downstream targets, thereby inhibiting tumor angiogenesis and growth in RCC.

TKI RESISTANCE

Resistance to a targeted agent develops when either the agent is no longer able to inhibit specific signaling pathways or when a tumor's ability to survive becomes independent from those pathways. Various mechanisms contribute to drug resistance, such as alterations in drug targets, activation of alternative signaling pathways, enhancement of drug efflux, and evasion of apoptotic cell death[26].

Resistance to antiangiogenic targeted therapies can be categorized as intrinsic (primary) or acquired (secondary)[27]. Intrinsic resistance is characterized by an inherent insensitivity of cancer cells to TKIs and a lack of response to the drug. By contrast, acquired resistance arises when cancer cells initially respond to TKI treatment but eventually relapse as the drug loses efficacy over time due to the acquisition of various resistance mechanisms. Multiple *in vitro* and *in vivo* models involving cell lines, patient-derived xenografts (PDX), organoids, and genetically engineered mouse models (GEMMs) have been developed to assess the mechanisms, dynamics, and progression of drug resistance[28]. These models help elucidate the complex interplay between cancer cells and their microenvironment, offering valuable information for developing strategies to overcome resistance and improve treatment outcomes. In the following section, we summarize the numerous mechanisms of TKI resistance identified to date in RCC.

ACQUIRED MECHANISMS OF TKI RESISTANCE IN RCC

Alternative proangiogenic pathways

Successful antiangiogenic TKI therapy suppresses the production of proangiogenic factors such as VEGF and PDGF and inhibits angiogenesis. This eventually leads to tumor hypoxia, which then triggers the upregulation of alternative proangiogenic pathways, contributing to eventual drug resistance. This process is also known as angiogenic escape or angiogenic switch.

One such pathway involves the increased expression of interleukin-6 (IL-6). IL-6 activates the STAT3 pathway, leading to the upregulation of HIF-2α and subsequent increased production of VEGFR[29]. IL-8 is also upregulated as a response to hypoxia and contributes to angiogenesis by promoting endothelial cell proliferation, survival, and migration via VEGF mRNA transcription and autocrine VEGFR-2 activation[30,31]. High levels of IL-6 and IL-8 have been correlated with significantly shorter PFS and OS in metastatic RCC patients treated with sunitinib and pazopanib[32,33].

Angiopoietin 1 and 2 (Ang 1/2) are critical regulators of angiogenesis, acting as ligands for the Tie2 receptor tyrosine kinase on endothelial cells[34]. When Tie2 is activated, it promotes vessel stabilization, survival, and maturation, thereby boosting the VEGF pathway's effectiveness in improving perfusion to RCC tumors[35]. Wang *et al.* followed Ang 2 levels as RCC patients were treated with sunitinib and found that Ang 2 decreased as patients responded to therapy, but then increased as patients became resistant and developed advanced disease[36].

C-Met, a tyrosine kinase encoded by the MET proto-oncogene, binds hepatocyte growth factor (HGF) and initiates an alternative proangiogenic pathway to VEGFR. Type 1 papillary RCC is commonly associated with activating MET alterations and has an unfavorable prognosis[37]. Inhibitors against c-Met have emerged as important treatment options for this disease in recent years. In the phase 2 CREATE trial by Schöffski *et al.*, patients with type 1 papillary RCC were categorized by MET mutation status and treated with crizotinib, a TKI against c-Met and ALK. Patients with MET alterations or amplifications were found to have a higher objective response rate, as well as increased PFS and OS compared to patients without MET alterations or amplifications[38]. High c-Met expression in ccRCC has been identified as an independent risk factor for higher tumor grade, aggressive phenotype, increased metastasis, and decreased overall survival[39].

C-Met activation and overexpression have been identified in ccRCC cells previously treated with anti-VEGFR TKIs, likely conferring resistance to these therapies[40]. Increased c-Met expression in patients previously treated with sunitinib has been correlated with shorter PFS and OS[41].

Other proangiogenic factors including fibroblast growth factors 1 and 2 (FGF 1/2) and ephrin A1 and A2 (EFNA 1/2) have also been found to be upregulated following VEGFR inhibition, likely as a result of tumor hypoxia[42]. The FGF receptor (FGFR) pathway regulates and activates alternative proangiogenic pathways such as mitogen-activated protein kinases/extracellular signal-related kinases (MAPK/ERK) and phosphoinositide-3-kinase/AK strain transforming signaling pathway (PI3K/Akt)[43].

Increased pericyte coverage

Pericytes are mural cells that surround the endothelial cells of blood vessels and promote vascular stability and angiogenesis[44]. PDGF-BB is secreted by vascular endothelial cells and binds to PDGFR-β on pericytes, activating signaling that leads to increased pericyte production of VEGF which promotes endothelial cell proliferation[45]. Increased pericyte activity in the tumor microenvironment has been associated with more aggressive ccRCC[46], and resistance to antiangiogenic TKIs has been observed in tumors with an increased number of pericytes[47]. A proposed mechanism of resistance is the inhibition of an important negative feedback loop. High VEGF levels lead to the formation of a VEGFR/PDGFR-β complex that suppresses PDGFR-β signaling, preventing the production of excessive VEGF[48]. In the setting of TKI therapy against VEGFR, this mechanism may be less effective, leading to overactive pericyte activity. Thus, all antiangiogenic TKIs induce resistance via this mechanism.

Multi-drug resistance via lysosomal sequestration and efflux transporters

Sunitib is a small, hydrophobic, weak base, which allows it to easily pass through the cellular membrane, accumulate intracellularly, and bind to the kinase domain of VEGFR[49]. However, this structure also lends itself to efficient passage into lysosomes, where a more acidic environment encourages protonation of the drug, conferring a positive charge and effectively sequestering it for degradation[50]. Although sunitinib is particularly susceptible due to its chemical properties, lysosomal sequestration has been identified as a contributor to multi-drug resistance (MDR) in cancer cells via a variety of mechanisms. Cancer cells have been found to upregulate PI3K, which promotes lysosomal activity and stability. Further, cancer cells have been found to abolish lysosomal membrane permeabilization (LMP), a process that normally induces apoptosis, via upregulation of cytosolic protease inhibitors and via increased translocation of Hsp70 to the lysosomal lumen, which stabilizes lysosomal membranes[51]. Following exposure to sunitinib, RCC cells exhibit a rise in lysosomal mass, facilitating enhanced sequestration[52]. Zhitomirsky *et al.* demonstrated that exposure of human carcinoma cells to sunitinib leads to an increase in the number of lysosomes per cell, as well as the number of lysosomes accumulating high levels of sunitinib[53].

Sunitinib exposure has also been associated with increased expression of ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-GP), which are present both on the lysosomal and cell membranes and promote both lysosomal sequestration and extracellular efflux[54]. Thus, lysosomal sequestration and efflux transporter proteins work synergistically to promote the development of resistance[55].

Bone marrow-derived cell recruitment

Bone marrow-derived cells (BMDCs) are a pool of progenitor cells, including hematopoietic stem cells, endothelial progenitor cells, tumor-associated macrophages, mesenchymal stromal cells, VEGFR1-positive hemangiocytes, and more[56]. Myeloid-derived suppressor cells (MDSCs) are a subpopulation of BMDCs that most commonly express CD11b and serve an immunomodulatory role in the tumor microenvironment, suppressing the activity of infiltrating cytotoxic T lymphocytes[57]. MDSCs produce

nitric oxide, which reacts with superoxide, generating peroxynitrite (PNT). PNT nitrates both T-cell receptors, reducing their responsiveness to antigen major histone compatibility (MHC) complexes, and T-cell specific chemokines, blocking T-cell migration[58]. MDSCs also produce proangiogenic proteins such as IL-8, MMP8, and MMP9[59]. MDSCs and other BMDCs are recruited to RCC tumors by various chemokines, promoting tumor angiogenesis and progression[60,61]. Sunitinib has been shown to reduce MDSCs in the peripheral blood of mRCC patients who had the local tumor resected[62]. However, persistently high levels of MDSCs have been found in cases of resistant RCC. This may be due to the increased production of granulocyte-macrophage colony-stimulating factor (GM-CSF), which protects MDSCs from sunitinib-induced apoptosis[59]. Recruitment of MDSCs and other BMDCs to the tumor microenvironment is likely due to hypoxia induced by TKI therapy. Therefore, though this effect of sunitinib is well-known, all approved antiangiogenic TKIs are thought to contribute to this process.

Tumor-associated fibroblasts

Tumor-associated fibroblasts (TAFs) are activated fibroblasts that have undergone phenotypic and functional changes in response to tumor-derived signals[63]. Unlike regular fibroblasts, TAFs remain chronically activated and continue to carry out their work indefinitely within the tumor microenvironment. They support tumor survival and growth by secreting various growth factors, cytokines, and chemokines, such as interleukins, HGF, and stromal cell-derived factor 1 alpha (SDF-1α)[64]. TAFs promote the deposition of a dense extracellular matrix, creating a physical barrier that hampers drug penetration into the tumor and supporting cell adhesion-mediated drug resistance[65,66]. Moreover, the role of TAFs in supporting cell adhesion-mediated drug resistance (CAM-DR) is well-defined. TAFs also play a part in reprograming tumor metabolism, making cancer cells less dependent on glucose, and increasing the lactate upload to drive anabolic pathways[64]. Platelet-derived growth factor-C (PDGF-C) mediates the angiogenic properties of TAFs and has been found to be upregulated in tumor cells resistant to anti-VEGF antibodies[67].

Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is an embryonic development process that can be hijacked by cancer cells to promote invasion, metastasis, and resistance to therapy. During EMT, epithelial cells lose characteristics such as cell-to-cell adhesion and apical-basal polarity, and acquire mesenchymal features including increased motility, invasiveness, and resistance to cell death[68]. This phenotypic switch is achieved by changes in gene expression, such as the downregulation of epithelial markers (E-cadherin) and upregulation of mesenchymal markers (N-cadherin, vimentin), as well as by epigenetic modifications[68]. EMT in cancer is largely driven by tumor hypoxia and resultant HIF-1α activation, as well as by inflammatory cytokines IL-6, IL-8, IL-15, and tumor necrosis factor-α (TNF-α)[20,69].

EMT is frequently discussed in the context of cancer stem cells (CSCs), which are subpopulations of cells within tumors that are capable of self-renewal and multi-lineage differentiation[70]. Because heterogeneous tumors contain many phenotypically distinct cells with varying degrees of response to chemotherapeutics, CSCs enhance drug resistance and tumor relapse. Processes associated with EMT, such as suppression of E-cadherin, have been shown to generate increased CSCs in tumors[71]. A specific side population of CSCs has been identified in RCC, and many RCC tumors express diverse markers associated with CSCs[72].

Acquired resistance to sunitinib via EMT has been demonstrated in patient-derived xenograft models. In a study by Hammers *et al.*, tumor tissue from skin metastases of a sunitinib-resistant ccRCC patient was implanted into nude mice, and these mice were treated with sunitinib or vehicle for 90 days. The average tumor volume was less than 200 mm^3 in mice treated with sunitinib and more than 800 mm^3 in mice treated with the vehicle, which suggests renewed sensitivity to sunitinib. The histology of the skin metastases indicated sarcomatous differentiation with a fibroblast-like appearance, indicating EMT. However, the cells

in the xenograft returned to normal ccRCC histology[73].

Pharmacogenomic factors

Single nucleotide polymorphisms (SNPs) are variations in a single nucleotide within the DNA sequence, which achieve an allelic frequency of at least 1% in a population, and they can affect gene expression, protein function, and drug metabolism[3]. Specific SNPs in drug-metabolizing enzymes, drug transporters, or drug targets can impact the efficacy of TKIs.

CYP3A4 and CYP3A5 are two of the principal enzymes responsible for sunitinib metabolism, converting the drug to its active metabolite, N-desethyl sunitinib (SU12662)[74]. SU12662 has a longer half-life than sunitinib, resulting in increased drug exposure[75]. The CYP3A4 SNP rs4646437G>A has been associated with an increased incidence of hypertension in mRCC patients treated with sunitinib. This is presumably due to enhanced sunitinib metabolism leading to increased concentrations of SU12662[76]. The expression of CYP3A4 is negatively regulated by two ligand-activated nuclear receptors, NR112 and NR113[77]. SNPs present in the genes encoding NR112 and NR113 have been associated with decreased patient PFS and OS, likely due to enhanced NR112 and NR113 activity leading to decreased CYP3A4 expression and lower SU12662 concentrations[78]. SNPs in CYP3A5, such as the CYP3A5*1 allele, have been associated with sunitinib toxicity and the need for dose reduction, likely due to enhanced CYP3A5 activity leading to increased concentrations of SU12662[79].

Additionally, SNPs in genes coding for ABC transporters have been found to affect the uptake and efflux of TKIs, altering drug concentrations within tumor cells. The presence of CGT in the ABCB1 haplotype is associated with improved PFS, likely due to decreased clearance of sunitinib and its active metabolite SU12662[79]. On the other hand, certain variants such as the TT-genotype in ABCB1 rs1125803 or the TT/TA-variant in ABCB1 rs2032582 have been found to promote drug efflux, resulting in increased intracellular clearance of sunitinib, increased time-to-dose reduction, and decreased PFS[80].

MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules that play a significant regulatory role in gene expression. miRNAs are known to contribute to cancer progression by silencing tumor suppressor genes via destruction or decreased expression of mRNA[81]. Multiple miRNAs have been implicated in mediating TKI resistance in RCC by targeting key signaling pathways involved in cell proliferation, survival, and drug response. For example, miR-15b has been found to be overexpressed in sunitinib-resistant cell lines after incubation with the drug. *In vivo* models have produced similar results[82]. Other notable miRNAs overexpressed in sunitinib-resistant RCC cells include miRNA-575, miRNA-642b-3p, and miRNA-4430 (all studied *in vitro*), as well as miRNA-942, miRNA-133a, miRNA-628-5p, and miRNA-484 (all studied *in vivo*)[83,84]. Additionally, miR-144-3p overexpression in ccRCC has been found to enhance cell proliferation, clonogenicity, migration, invasion, and resistance via repression of the tumor suppressor gene, ARID1A[85].

In contrast, miR-200b and miR-141 have been found to be downregulated in ccRCC compared to benign tissue, and their expression may represent an independent prognostic factor for increased PFS and OS[86]. Figure 2 illustrates the wide array of known acquired mechanisms of TKI resistance in RCC.

INTRINSIC MECHANISMS OF TKI RESISTANCE IN RCC

Methylation of tumor suppressor genes

Enhancer of zeste homolog 2 (EZH2) is an enzyme that functions as the catalytic subunit of the polycomb

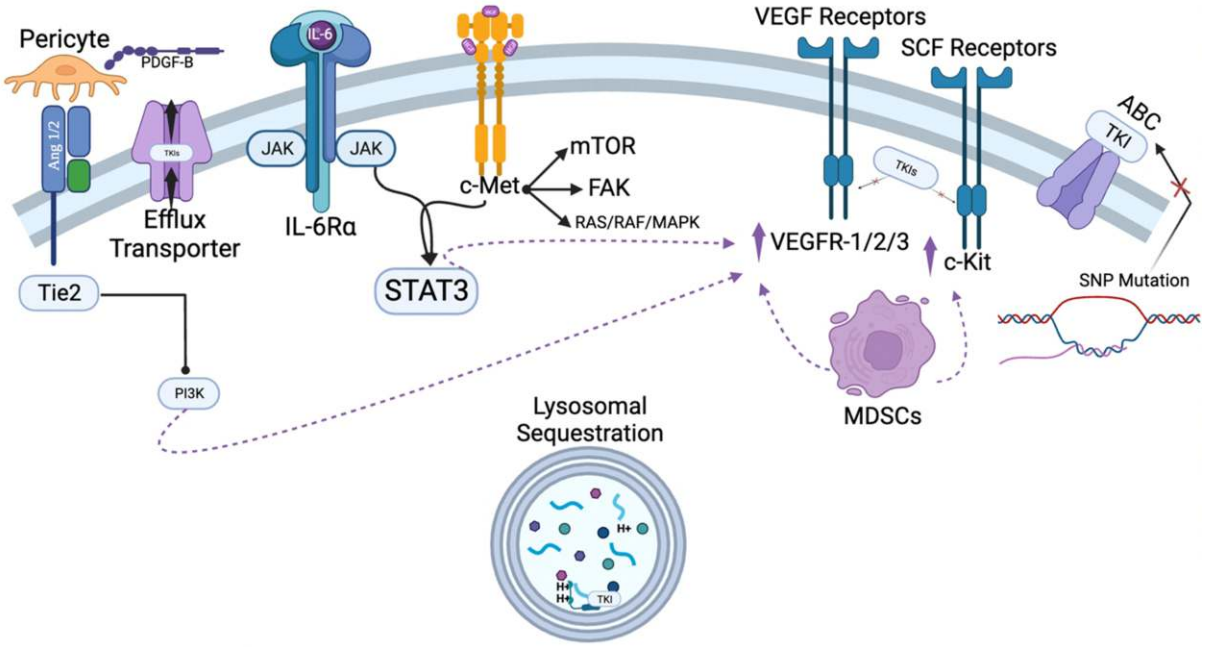

Figure 2. Common acquired mechanisms of resistance to tyrosine kinase inhibitors in RCC. ABC: ATP-binding cassette; IL-6: interleukin-6; MAPK: mitogen-activated protein kinases; MDSCs: myeloid-derived suppressor cells; mTOR: mammalian target of rapamycin; PDGF: platelet-derived growth factor; PI3K: phosphoinositide-3-kinase; RCC: renal cell carcinoma; SNP: single nucleotide polymorphism; TKIs: tyrosine kinase inhibitors; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

repressive complex 2 (PRC2). Its primary function is to methylate lysine 27 on histone H3, resulting in gene silencing and transcriptional repression[87]. Intrinsic increased expression of EZH2 can lead to aberrant methylation of specific tumor suppressor genes, rendering them inactive. Methylation of these genes can disrupt critical cellular pathways involved in growth control and DNA repair, leading to the promotion of tumorigenesis. The dysregulation of EZH2-mediated methylation can confer resistance to TKIs and other antineoplastic agents by circumventing the inhibitory effects of these drugs on oncogenic signaling pathways. The overexpression of EZH2 has been reported in RCC and is associated with poor prognosis[88,89].

Inhibition of apoptosis

B cell lymphoma-2 (Bcl-2) and B cell lymphoma-extra large (Bcl-xL) are proteins within the Bcl-2 family and play a key role in inhibiting apoptosis. These are known to be upregulated in many cancers, contributing to proliferation and metastasis[90]. There is limited evidence that overexpression of these proteins may confer intrinsic resistance to TKIs and other antineoplastic agents in RCC via inhibition of apoptosis, though further study is needed[91]. Figure 3 demonstrates known intrinsic mechanisms of resistance to TKIs in RCC.

OVERCOMING TKI RESISTANCE IN RCC

Effective mitigation of TKI resistance plays a vital role in improving outcomes for patients with advanced RCC. Undoubtedly, this is a significant challenge due to the diverse range of mechanisms through which RCC can develop resistance. However, the multitude of mechanisms also offers numerous avenues for

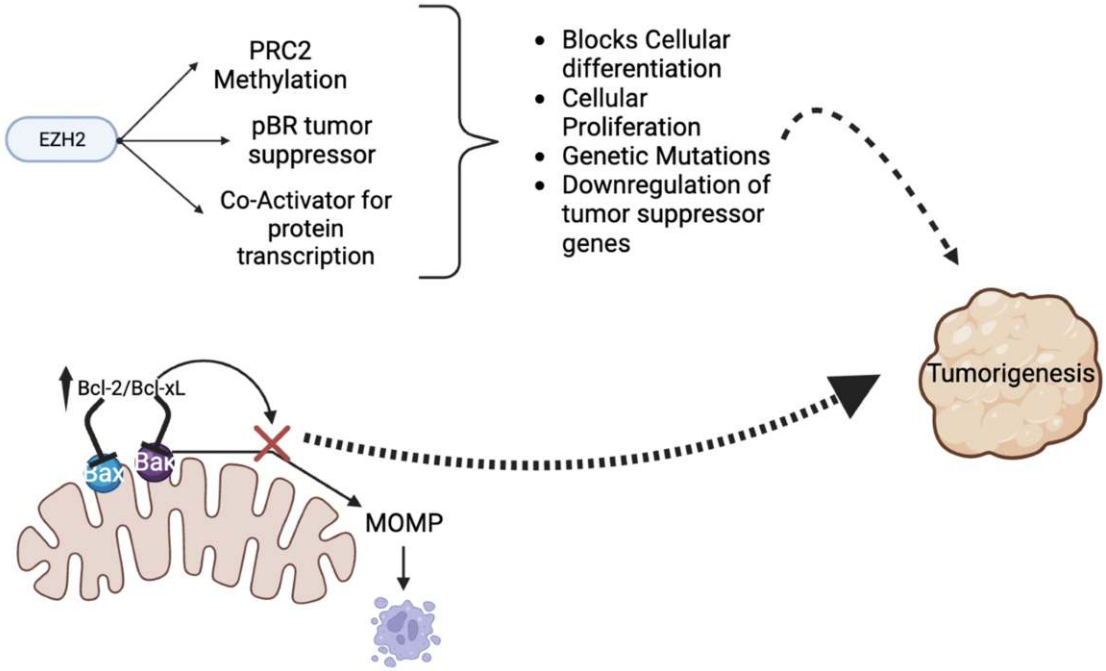

Figure 3. Intrinsic resistance mechanisms affecting tyrosine kinase inhibitor activity in RCC. Bcl-2: B cell lymphoma-2; Bcl-xL: B cell lymphoma-extra large; EZH2: enhancer of zeste homolog 2; PRC2: polycomb repressive complex 2; RCC: renal cell carcinoma.

intervention, enabling the targeting of various processes to minimize resistance. Table 1 summarizes the many resistance mechanisms herein described, as well as potential approaches for overcoming these mechanisms.

One strategy is to target processes upstream from VEGF/VEGFR, such as HIF-2α. In hypoxic conditions, HIF-2α promotes the expression of multiple hypoxia-inducible genes, including VEGF and PDGF-BB. As previously discussed, in VHL syndrome, the negative regulatory protein pVHL is lost, leading to the accumulation of HIF-2α and excessive angiogenesis[24]. HIF-2α inhibitors act by inhibiting the dimerization of HIF-2α and its partner protein, ARNT1, thereby inhibiting HIF-2α mediated transcription[92]. The HIF-2α inhibitor, belzutifan, was approved by the FDA for the treatment of germline VHL-mutated RCC after a phase 2 trial demonstrated promising efficacy in this population[93]. Other studies examining belzutifan in sporadic (non-VHL associated) RCC are also encouraging[94]. In a phase 1 expansion cohort, belzutifan in combination with nivolumab was administered to patients with advanced ccRCC, and patients with therapeutic exposure to the drug had a median PFS of 10.0 months compared to just 4.7 months for patients with subtherapeutic exposure[95]. An ongoing trial evaluating the combination of belzutifan and cabozantinib is currently underway[96].

Directly targeting the alternative proangiogenic pathways upregulated during resistance development may also be effective. IL-6 and IL-8, both upregulated in the setting of tissue hypoxia following effective antiangiogenic therapy, are prime examples[47]. These cytokines can induce a cascade of immunologic changes that promote angiogenesis. Interestingly, co-administration of IL-8 neutralizing antibody has shown promise in re-sensitizing xenograft tumors to sunitinib treatment[97]. Administration of the anti-IL-6 antibody, tocilizumab, given in combination with interferon, has also slowed RCC xenograft proliferation[29]. The Ang/Tie signaling pathway, which augments the VEGFR pathway and promotes blood vessel stabilization, survival, and maturation, can be directly targeted with the TKI trebananib[98]. In

Table 1. Acquired and intrinsic mechanisms of tyrosine kinase inhibitor resistance in RCC

Process	Mechanism	TKIs affected	Potential therapies	Ref.
Alternative proangiogenic pathways	Multiple; tumor hypoxia induces upregulation of interleukins, c-Met/HGF, angiopoietins, and growth factors	Sunitinib, pazopanib, sorafenib, axitinib, tivozanib	HIF-2α inhibitors; anti-IL-6 and IL-8 antibodies; TKIs against Ang 1 and 2, c-Met, and FGFR	[29-43, 92-104]
Increased pericyte coverage	Increased tumor endothelial stability and production of VEGF	Sunitinib, pazopanib, sorafenib, axitinib, tivozanib	Unknown	[44-48]
Lysosomal sequestration	Drug sequestered; subtherapeutic intracellular concentrations; ineffective inhibition of receptor tyrosine kinases	Sunitinib	Alkalizing agents; drugs against lysosomal membrane proteins	[49-53, 55,108]
Efflux pumps	Subtherapeutic intracellular concentrations	Sunitinib known, but other TKIs possible	P-GP inhibitors	[54,55, 109-113]
Bone marrow-derived cell recruitment	Bone marrow-derived cells accumulate around tumor; immunomodulatory effects including suppression of cytotoxic T cells	Sunitinib, pazopanib, sorafenib, axitinib, tivozanib	Immune checkpoint inhibitors to rejuvenate immune response in the tumor microenvironment	[56-62]
Tumor-associated fibroblasts	Overactive fibroblasts secrete interleukins, hepatocyte growth factor, and more in tumor microenvironment; increased extracellular matrix deposition hampers drug penetration and supports cell adhesion	Unknown, but all TKIs possible	Histone deacetylase inhibitors	[63-67, 105,107]
Epithelial-mesenchymal transition	Tumor hypoxia drives epithelial cells to acquire features such as increased motility, invasiveness, and resistance to cell death	Sunitinib, pazopanib, sorafenib, axitinib, tivozanib	Drugs with activity against EMT in combination with antineoplastic agents	[20,68 -72,105, 106]
Pharmacologic Factors	Single nucleotide polymorphisms in genes related to sunitinib metabolism lead to decreased concentrations of active metabolite	Sunitinib	Unknown	[3,74-80]
miRNAs	Silencing of tumor suppressor genes via destruction or decreased expression of mRNA	Sunitinib	Unknown	[81-86]
Methylation of tumor suppressor genes	EZH2 overexpression leads to aberrant methylation of tumor suppressor genes, rendering them inactive	All antineoplastic and targeted drugs	EZH2 inhibitors; hypomethylating agents	[87-89, 114]
Inhibition of apoptosis	Bcl-2 and Bcl-xL overexpression inhibits tumor cell apoptosis	All antineoplastic and targeted drugs	Bcl-2 inhibitors	[90,91, 115]

Bcl-2: B cell lymphoma-2; Bcl-xL: B cell lymphoma-extra large; EMT: epithelial-mesenchymal transition; EZH2: Enhancer of zeste homolog 2; FGFR: fibroblast growth factors receptor; HGF: hepatocyte growth factor; IL-6: interleukin-6; IL-8: interleukin-8; miRNAs: MicroRNAs; P-GP: P-glycoprotein; RCC: renal cell carcinoma; TKIs: tyrosine kinase inhibitors; VEGF: vascular endothelial growth factor.

xenograft mouse models, treatment with trebananib in combination with sunitinib slowed tumor progression compared to treatment with sunitinib plus control[36]. However, other results have been less promising. A phase 2 trial examined the efficacy of trebananib with or without continued anti-VEGF therapy in RCC patients who had previously progressed on anti-VEGF therapy and observed poor outcomes in both treatment arms[99]. Targeting c-Met in cells that have become resistant to TKI's against VEGF may also be effective. Studies have shown that the growth of sunitinib-resistant tumors can be slowed with a combination of sunitinib and a c-Met inhibitor[39]. Likewise, Zhou *et al.* found that treatment with cabozantinib, a TKI against VEGFR and MET, can rescue acquired sunitinib resistance in xenograft mouse models[100]. Finally, fibroblast growth factor (FGF) overexpression, specifically FGF2, has been associated with a poor RCC prognosis and contributes to TKI resistance through suppression of antiangiogenic activity[42,101]. There are several multitargeted TKIs that inhibit FGFR, such as lenvatinib, but these drugs are themselves susceptible to resistance[102]. *In vitro* studies have suggested that combining FGFR inhibitors with other TKIs such as PI3K inhibitors may help mitigate this resistance and inhibit RCC cell metabolic activity[103]. PI3K inhibitors have also demonstrated strong *in vitro* anti-tumor activity when co-administered with sunitinib[104].

Targeting proangiogenic changes in the tumor microenvironment may be useful in combatting TKI resistance. The process of EMT, which promotes resistance to therapy and metastases, is associated with a host of metabolic changes. Although there are no direct inhibitors to EMT, multiple metabolism-inhibiting drugs have shown indirect activity against EMT changes through various complex biochemical mechanisms[105]. There are multiple ongoing clinical trials examining the combination of these drugs with antineoplastics agents, although none of these trials include RCC patients[106]. Additionally, TAFs may serve as a target for overcoming resistance. Histone deacetylase inhibitors offer a promising approach to diminish the activation of TAFs and eradicate their infiltration within the tumor stroma, as demonstrated by a study in patients with relapsed or refractory lymphoma or multiple myeloma[105,107].

Lysosomal sequestration of TKIs is another potentially reversible mechanism of resistance. Combination therapies incorporating alkalizing treatments have been explored, creating a toxic environment that counteracts the sequestration process[51]. Drugs targeting various lysosomal membrane proteins have shown promise in destabilizing the lysosomal membrane, leading to membrane permeabilization[55]. Interestingly, Gotink *et al.* showed that sunitinib efficacy in RCC cells that had developed resistance via lysosomal sequestration can be restored by culturing resistant cells with sunitinib-free media[108]. This supports lysosomal sequestration as an acquired, transient, and reversible resistance mechanism.

Additionally, RCC has been shown to overexpress the ABC transporter P-glycoprotein (P-GP) in response to hypoxia from antiangiogenic therapy. P-GP is predominantly found in the cell membrane and exports drugs, contributing to multi-drug resistance[109,110]. P-GP is also commonly found in enterocytes and contributes to resistance via drug export into the GI lumen, limiting drug oral bioavailability[111]. Several studies have examined the co-administration of inhibitors to P-GP with anticancer drugs in various malignancies, but this strategy has been limited by severe toxicity and unwanted side effects[112]. Despite evidence of *in vitro* anti-tumor activity, there are currently no P-GP inhibitors approved for cancer treatment[113].

Intrinsic mechanisms may also be targeted to overcome resistance. EZH2 overexpression in RCC contributes to hypermethylation, and ultimately inactivation, of tumor suppressor genes. The efficacy of EZH2 inhibitors and hypomethylating agents is currently not defined in many solid tumors, including RCC, but investigative studies are underway[114]. Antiapoptotic proteins such as Bcl-2 and Bcl-xL also confer intrinsic resistance. There is limited evidence regarding the use of Bcl-2 inhibitors in RCC. However, one preclinical study has demonstrated a potential synergetic effect of the Bcl-2 inhibitor, venetoclax, when given sequentially prior to sunitinib[115].

In recent years, ICIs targeting programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) have emerged as a cornerstone in the treatment of advanced RCC. These therapies block interactions between immune checkpoint proteins, thereby triggering immune activation against various cancers[116]. ICIs are frequently combined with TKIs to leverage their synergistic effects resulting from complementary mechanisms of action. Notably, all TKI resistance mechanisms share a common thread of promoting angiogenesis. Proangiogenic molecules like VEGF impede both the innate and adaptive immune systems by hindering precursor cell differentiation, upregulating PD-1, PD-L1, and CTLA-4 on immune cells, and recruiting MDSCs[117]. ICI therapy rejuvenates the immune response, counteracting these immunosuppressive effects and mitigating resistance.

CONCLUSION

TKIs have significantly improved the outcomes of patients with advanced RCC by targeting key pathways involved in cancer proliferation, survival, and metastasis. However, treatment with VEGF-targeted TKIs is almost always characterized by the eventual development of resistance and subsequent disease progression. Mitigating drug resistance is challenging due to the diverse mechanisms underlying TKI resistance, including upregulation of alternative proangiogenic pathways, EMT, efflux pumps reducing intracellular drug concentrations, lysosomal sequestration, alterations in the tumor microenvironment, and genetic factors.

Understanding these mechanisms is crucial for the development of innovative therapeutic approaches to overcome TKI resistance. Notably, the combination of TKIs with other agents, particularly ICIs, has made significant strides towards accomplishing this aim, solidifying ICIs as a cornerstone in the treatment of advanced RCC. Additionally, strategies to reverse or inhibit specific resistance mechanisms hold the potential to restore TKI efficacy. The continued exploration of combination therapies, comprehensive understanding of the tumor microenvironment, and identification of additional genetic biomarkers will pave the way for more effective treatments, ultimately improving outcomes for patients with advanced RCC.

DECLARATIONS

Authors' contributions

Investigation, writing, reviewing and editing: Sweeney PL
Investigation, writing: Suri Y
Supervision, reviewing and editing: Basu A
Supervision, reviewing and editing: Koshkin VS
Project conceptualization, supervision, reviewing and editing: Desai A

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Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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AUTHOR INSTRUCTIONS

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The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Word Limit	Abstract	Keywords	Main Text Structure

Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	5000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	7000 max	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author include an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	2500 max	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	5000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	3000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	3500 max	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	2500 max	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	1000 max	None required	None required	/
Letter to Editor	A Letter to Editor is usually an open post-publication review of a paper from its readers, often critical of some aspect of a published paper. Controversial papers often attract numerous Letters to Editor	1000 max	Unstructured abstract (optional). No more than 250 words.	3-8 keywords (optional)	/
Opinion	An Opinion usually presents personal thoughts, beliefs, or feelings on a topic.	1200 max	Unstructured abstract (optional). No more than 250 words.	3-8 keywords	/
Perspective	A Perspective provides personal points of view on the state-of-the-art of a specific area of knowledge and its future prospects. Links to areas of intense current research focus can also be made. The emphasis should be on a personal assessment rather than a comprehensive, critical review. However, comments should be put into the context of existing literature. Perspectives are usually invited by the Editors.	2000 max	Unstructured abstract. No more than 150 words.	3-8 keywords	/

2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

2.3.1.2 Authors and Affiliations

Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

2.3.1.3 Abstract

Original research, systematic reviews, and meta-analyses require structured abstracts. The abstract should provide the context or background for the study and should state the study's purpose, basic procedures (selection of study participants, settings, measurements, analytical methods), main findings (giving specific effect sizes and their statistical and clinical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations, note important limitations, and not overinterpret findings. Clinical trial abstracts should include items that the CONSORT group has identified as essential. It is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

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The graphical abstract is essential as this can catch first view of your publication by readers. We request the authors submit an eye-catching figure during the revision stage. It should summarize the content of the article in a concise graphical form. It is recommended to use it because this can make online articles get more attention. The graphic abstract should be submitted as a separate document in the online submission system along with the revised version. Please provide an image with a minimum of 730 × 1,228 pixels (h × w) or proportionally more. The image should be readable at a size of 7 × 12 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, PSD, AI, JPG, JPEG, EPS, PNG, ZIP and PDF files.

2.3.1.5 Keywords

Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

2.3.2 Main Text

Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

2.3.2.1 Introduction

The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

2.3.2.2 Methods

Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

2.3.2.3 Results

This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

2.3.2.4 Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

2.3.2.5 Conclusion

It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

2.3.3 Back Matter

2.3.3.1 Acknowledgments

Anyone who contributed towards the article but does not meet the criteria for authorship, including those who provided professional writing services or materials, should be acknowledged. Authors should obtain permission to acknowledge from all those mentioned in the Acknowledgments section. This section is not added if the author does not have anyone to acknowledge.

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Please use Surname and Initial of Forename to refer to an author's contribution. For example: made substantial contributions to conception and design of the study and performed data analysis and interpretation: Salas H, Castaneda WV; performed data acquisition, as well as provided administrative, technical, and material support: Castillo N, Young V.

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Studies involving animals and cell lines must include a statement on ethical approval. More information is available at Editorial Policies.

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2.3.3.9 References

Preferably original research articles that directly support the statements should be cited. Review articles could be cited

when they specifically address the statement made in the manuscript. An abstract should not be used as a reference. Non-specific citations should be avoided.

References should be numbered in order of appearance at the end of manuscripts. In the text, reference numbers should be placed in square brackets and the corresponding references are cited thereafter. If the number of authors is less than or equal to six, we require to list all authors' names. If the number of authors is more than six, only the first three authors' names are required to be listed in the references, other authors' names should be omitted and replaced with "et al.". Abbreviations of the journals should be provided on the basis of Index Medicus. Information from manuscripts accepted but not published should be cited in the text as "Unpublished material" with written permission from the source.

References should be described as follows, depending on the types of works:

Types	Examples
Journal articles by individual authors	Weaver DL, Ashikaga T, Krag DN, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med* 2011;364:412-21. [PMID: 21247310 DOI: 10.1056/NEJMoa1008108]
Organization as author	Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002;40:679-86. [PMID: 12411462]
Both personal authors and organization as author	Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1,274 European men suffering from lower urinary tract symptoms. *J Urol* 2003;169:2257-61. [PMID: 12771764 DOI: 10.1097/01.ju.0000067940.76090.73]
Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, Editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

For other types of references, please refer to U.S. National Library of Medicine.
The journal also recommends that authors prepare references with a bibliography software package, such as EndNote to avoid typing mistakes and duplicated references.

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Additional data and information can be uploaded as Supplementary Materials to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English, and limited to a size of 500 MB.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

The word limit is specified in the item "Types of Manuscripts". There are no restrictions on number of figures or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

Manuscripts must be written in English.

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Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;
Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;
Figure caption is placed under the Figure;
Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, *etc.*) should be editable in word, excel or powerpoint format. Non-English information should be avoided;
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Abbreviations and symbols used in table should be explained in footnote;
Explanatory matter should also be placed in footnotes;
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2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc.*, can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

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SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

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Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

2.4.11 Equations

Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

2.5 Submission Link

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3. Research and Publication Ethics

3.1 Research Involving Human Subjects

All studies involving human subjects must be in accordance with the Helsinki Declaration and seek approval to conduct the study from an independent local, regional, or national review body (e.g., ethics committee, institutional review board, *etc.*). Such approval, including the names of the ethics committee, institutional review board, *etc.*, must be listed in a declaration statement of Ethical Approval and Consent to Participate in the manuscript. If the study is judged exempt from ethics approval, related information (e.g., name of the ethics committee granting the exemption and the reason for the exemption) must be listed. Further documentation on ethics should also be prepared, as editors may request more detailed information. Manuscripts with suspected ethical problems will be investigated according to COPE Guidelines.

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A case report is considered the diagnosis, treatment and post-treatment follow-up of a single patient. A case series is considered a group of case reports involving patients who were all given similar treatments. A clinical dataset is a list of well-defined variables collected during ongoing patient care or as part of a clinical trial program. It includes electronic health records, administrative data, patient registries, and clinical trial data.

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Authors must keep data anonymized. If participants' details are not to be anonymized, authors must ensure that written informed consent, including consent for publication, was obtained from each participant, and consent statement must be included in the manuscript.

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9. Editorial Process

9.1 Initial check

9.1.1 Initial manuscript check

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