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Cancer Drug Resistance

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Editor's Note

Cancer therapy resistance remains a major barrier to durable clinical responses across solid tumors and hematologic malignancies. This section brings together a curated selection of studies published in Cancer Drug Resistance that focus on clinically relevant mechanisms of therapeutic failure and strategies to overcome them. The highlighted articles span antibody–drug conjugate (ADC) resistance, chemotherapy failure in colorectal cancer, targeted therapy resistance in KRAS-mutant disease, and apoptosis dysregulation in treatment-refractory tumors.

Collectively, these works emphasize how resistance emerges in direct response to therapeutic pressure in clinically meaningful contexts, including HER2-positive breast cancer, colorectal cancer, non-small cell lung cancer, and glioblastoma. Mechanistic insights into drug efflux transporters, signaling rewiring, tumor microenvironment remodeling, and lineage-specific transcriptional dependencies provide a framework for translating laboratory discoveries into improved therapeutic strategies.

By integrating pharmacological resistance mechanisms with disease-specific biology, this section underscores the evolving interface between cancer treatment and resistance biology. It highlights how understanding therapy-driven adaptation is essential for optimizing current regimens and guiding the development of next-generation anticancer agents.

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Mechanisms of action and resistance to anti-HER2 antibody-drug conjugates in breast cancer

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Mechanism of Resistance		Overcoming resistance
Antigen-related resistance	• Reduced HER2/NECTIN4 levels • Tumor heterogeneity • Truncated forms of antigen ectodomain • Parallel genomic alteration • Heregulin	• Bystander effect • Biparatropic ADC
Payload-related resistance	• DAR • Payload conjugation	• Payload diversification: replacement of payload • Lysine vs. cysteine conjugate
Altered internalization	• Microenvironment and enzymatic role	
Impaired lysosomal function	• Lysosomal pH	• Replacement of hydrophobic with hydrophilic linkers
Overexpression of drug-efflux pumps	• ATP-binding cassette (ABC) transporters	• Aberrant expression of transporters • Transporter inhibition
Activation of signaling pathways	• PI3K/AKT signaling activation • Wnt/β-catenin pathway	• Combination therapies

Abstract

Human epidermal growth factor 2 (HER2)-positive breast cancer (BC) represents nearly 20% of all breast tumors. Historically, these patients had a high rate of relapse and dismal prognosis. The advent of HER2-targeting monoclonal antibodies such as trastuzumab followed by pertuzumab had improved the prognosis of HER2-positive metastatic BC. More recently, antibody-drug conjugates (ADCs) are now reshaping the treatment paradigm of solid tumors, especially breast cancer. Trastuzumab emtansine (T-DM1) was one of the first ADC developed in oncology and was approved for the management of HER2-positive metastatic BC. In a head-to-head comparison, trastuzumab deruxtecan (T-DXd) defeated T-DM1 as a second-line treatment. The efficacy of ADCs is counterbalanced by the appearance of acquired resistance to these agents. In this paper, we summarize the mechanisms of action and resistance of T-DM1 and T-DXd, as well as their clinical efficacy. Additionally, we also discuss potential strategies for addressing resistance to ADC.



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INTRODUCTION

Breast cancer (BC) is the most common cancer in women worldwide and the second leading reason of cancer-related mortality. It accounts for 287,000 new diagnosed cases and approximately 43,000 deaths in the United States in 2022^[1]. Human epidermal growth factor 2 (HER2)-positive BC represents nearly 20% of newly diagnosed patients. The HER2-neu gene encodes an oncoprotein that belongs to the epidermal growth factor receptor (EGFR) family consisting of four members^[2-4]. This subtype of tumors has historically been linked to an aggressive phenotype, a higher rate of relapse, and a worse prognosis compared to other tumor subtypes if untreated^[5]. This worse prognosis of HER2-positive BC has dramatically ameliorated with the development of anti-HER2 monoclonal antibodies (mAb). Trastuzumab, in combination with chemotherapy, was the first mAb approved in 1998 in HER2-positive metastatic BC^[6]. Trastuzumab was also approved as a neoadjuvant and adjuvant treatment in combination with chemotherapy. Pertuzumab is another HER2-targeting recombinant humanized mAb. The combination of chemotherapy, trastuzumab, and pertuzumab represents the gold standard in first-line setting in metastatic HER2-positive BC based on the CLEOPATRA study^[7]. The combination regimen has been then approved as a neoadjuvant treatment based on the NeoSphere phase II trial results^[8]. Tyrosine kinase inhibitors (TKIs) targeting HER2, such as tucatinib, lapatinib, and neratinib, were also incorporated into the therapeutic arsenal of patients with metastatic BC^[9]. The activity of all these drugs was limited by the development of resistance to HER2-targeting agents, and patients presented with disease progression. Antibody-drug conjugates (ADCs) are an emerging category of treatment developed in order to prevent or delay the emergence of resistance to treatment and to ameliorate efficacy in patients with metastatic BC. The first approved ADC was trastuzumab emtansine (T-DM1) as a second-line treatment in this subgroup of patients according to the randomized phase III EMILIA study in 2013^[10,11]. Then came trastuzumab deruxtecan (T-DXd), a new-generation ADC, to replace T-DM1 in the second-line setting according to the results of a first randomized DESTINY-Breast03 phase III trial comparing T-DXd vs. T-DM1^[12,13]. [Table 1](#) summarizes the approvals of T-DM1 and T-DXd. Once again, the clinical efficacy of ADC is counterbalanced by the occurrence of acquired resistance. In this review, we focus on the structure of ADC, the mechanism of action and the clinical efficacy of T-DXd and T-DM1. We also discuss the mechanisms of resistance to these agents, and the strategies to overcome such resistance.

STRUCTURE OF ADC

ADCs are composed of humanized mAbs binding to an antigen specific to the tumor and a payload (cytotoxic drug) via a linker that could be cleavable or non-cleavable^[14]. The antibodies incorporated in ADCs are fully humanized and most commonly IgG1-based because of their easier fabrication and stronger immunogenic characteristics in comparison with other IgG subtypes^[15]. The antibody contains two fragments that bind to antigens, known as Fabs, and a constant fragment (Fc). Fabs mediate the identification of the antigen, while Fc is responsible for the interaction between the antibody and the effector immune cells^[16]. An optimal antibody might present high affinity of binding to the correspondent target with low immunogenicity and good internalization with a relatively long plasma half-life^[17]. Activation of immune cells, activation of the complement complexes, induction of complement-dependent cytotoxicity (CDC), activation of antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis (ADCP) represent the main immune effector function of the antibody^[18].

The payload (cytotoxic drug) is very potent and represents the most important element of the ADC. Cytotoxic payloads are divided into several categories such as anti-tubulin (maystatine analogs, auristatin analogs), topoisomerase I inhibitors (deruxtecan, govitecan), DNA-damaging agents (calicheamicins, duocarmazine, pyrrolobenzodiazepines), and RNA polymerase II inhibitor (amanitin). These payloads had an unfavorable therapeutic index if given intravenously as non-conjugated drugs^[19].

Table 1. Current approvals of T-DM1 and T-DXd

Drug	Study name	Date of approval	Indication
T-DM1	EMILIA	February 2013	HER2+ MBC previously treated with taxane and trastuzumab
T-DM1	KATHERINE	May 2019	Adjuvant treatment in HER2+ EBC with residual disease after NAC with taxane and trastuzumab
T-DXd	DESTINY-Breast01	December 2019	HER2+ MBC after two treatment lines
T-DXd	DESTINY-Gastric01	January 2021	HER2+ MGC or GEJ after a prior trastuzumab-based treatment
T-DXd	DESTINY-Breast03	May 2022	HER2+ MBC in second-line treatment
T-DXd	DESTINY-Breast04	August 2022	HER2-low MBC after prior systemic treatment
T-DXd	DESTINY-Lung02	August 2022	HER2-mutant metastatic NSCLC

T-DM1: Trastuzumab emtansine; T-DXd: trastuzumab deruxtecan; MBC: metastatic breast cancer; EBC: early breast cancer; NAC: neoadjuvant chemotherapy; MGC: metastatic gastric cancer; GEJ: gastroesophageal junction; NSCLC: non-small cell lung cancer.

The antibodies are connected to the cytotoxic payloads via linkers that lead to the stabilization of the ADCs and affect the pharmacokinetic characteristics of these drugs. These linkers might be cleavable, a process linked to factors specific to the tumors, such as pH changes (acid pH), reduction-oxidation conditions, or lysosomal enzymes. They can also be non-cleavable and require more processing to release cytotoxic payloads. In the case of non-cleavable linkers, the whole proteolytic degradation of the antibody happens in the lysosomes, a mechanism that might influence the payload. However, cleavable linkers presented lower plasma stability and shorter half-lives compared to non-cleavable linkers^[20-22].

Drug-to-antibody ratio (DAR) is an important indicator and characteristic of ADCs. It refers to the number of drug molecules linked to the antibody. In general, the potency of ADCs is positively correlated to DAR. High DAR could be associated with quick clearance of the drug and, probably, higher toxicity. In contrast, lower DAR could be associated with decreased activity of ADC and increased therapeutic index^[23,24].

MECHANISM OF ACTION AND CLINICAL ACTIVITY

T-DM1

As previously mentioned, T-DM1 is the first anti-HER2 ADC approved for the management of metastatic BC. It is composed of trastuzumab as HER2-targeting IgG1 monoclonal antibody and linked to emtansine (also known as DM1), a microtubule-inhibitory agent derivative of maytansine, via a non-cleavable linker. T-DM1 has a DAR of 3.5^[21]. The mechanism of action of T-DM1 is complex. It is based on selective liberation of emtansine to HER2-positive tumor cells, inhibition of HER2 signaling by the trastuzumab, and shedding of the HER2 extracellular domain. T-DM1 can also evoke ADCC, similar to trastuzumab^[25-27]. Another mechanism of action of T-DM1 that has been reported by Hunter *et al.* was the mitotic catastrophe that inhibits tumor growth with prolonged treatment of HER2-positive xenografts with T-DM1. It is characterized by the presence of a high proportion of tumor cells with aberrant mitotic figures^[27]. The clinical activity of T-DM1 was evaluated in the EMILIA phase III study. It showed the superiority of T-DM1 over capecitabine combined with lapatinib in second-line treatment. T-DM1 had statistically significant higher median PFS (9.6 vs. 6.4 months; $P < 0.001$) and median OS (30.9 vs. 25.1 months; $P < 0.001$). In 2013, the FDA approved T-DM1 as a second-line treatment for the management of patients with HER2-positive metastatic BC, according to the results of this study^[10,11]. However, T-DM1 failed to be positioned as a first-line treatment in the MARIANNE phase III study that randomized T-DM1 alone or with pertuzumab vs. taxane and trastuzumab. T-DM1 was not superior to control group^[28]. Moreover, the incorporation of T-DM1 in the neoadjuvant treatment was not successful in the KRISTINE trial that compared T-DM1 in combination with pertuzumab vs. docetaxel, carboplatin, trastuzumab, and pertuzumab^[29,30]. More recently, T-DM1 has been positioned in adjuvant treatment in patients who had remaining residual disease following anti-HER2-based neoadjuvant treatment based on the results of the KATHERINE phase III study. T-DM1 significantly reduced the risk of relapse or death by 50% in comparison with trastuzumab alone^[31]. In this trial, the most frequent adverse events (AEs) contributing to T-DM1 discontinuation were

thrombocytopenia (4.2%), increased bilirubin level (2.6%), and transaminase elevation (1.6%). The benefit of adjuvant T-DM1 is demonstrated in this subgroup of patients despite liver toxicity leading to treatment discontinuation in 1% to 2% of patients^[31].

T-DXd, DS8201

T-DXd is a third-generation ADC composed of trastuzumab, linked to deruxtecan, a potent topoisomerase I inhibitor and is an exatecan derivative, via a cleavable linker^[32]. T-DXd is characterized by multiple features such as its potent payload, short systemic half-life avoiding systemic exposure, a DAR of 8, and the bystander effect^[33]. The phase II DESTINY-Breast01 clinical trial investigated the dosage of 5.4 mg/kg of T-DXd every three weeks, in pretreated patients with HER2-positive metastatic BC and resistant to T-DM1. The clinical activity of T-DXd was confirmed with an ORR of 61% and the median PFS was 19.4 months. Interstitial lung disease (ILD) was reported in nearly 15% of patients and two patients died^[34]. Based on these results, the FDA and the EMA approved T-DXd for the treatment of patients with HER2-positive metastatic BC who had received two or more anti-HER2-based regimens including T-DM1. More recently, the DESTINY-Breast02 confirmed the superiority of T-DXd over trastuzumab or lapatinib in combination capecitabine in patients with HER2-positive metastatic BC who had experienced treatment failure with T-DM1, with no additional safety concerns. The median PFS was 17.8 months in the T-DXd group *vs.* 6.9 months in the control arm ($P < 0.0001$)^[35]. Ultimately, T-DXd was directly compared with T-DM1 in the DESTINY-Breast03 phase III in patients with metastatic BC previously treated with taxane and trastuzumab. The study met its primary endpoint of PFS. In this setting, T-DXd dethroned T-DM1 with impressive results in terms of PFS and OS (that was a secondary endpoint). The median PFS was 28.8 months with T-DXd *vs.* 6.8 months for T-DM1 patients ($P < 0.0001$). The median OS was not reached in both arms at a median follow-up of nearly 2 years with a HR of 0.64 ($P = 0.0037$) in favor of T-DXd^[12,13]. Once again, the most frequent grade 3 or more treatment-related adverse events (TRAEs) were neutropenia (19.1%) and thrombocytopenia (7%). ILD was reported in 10.5% of patients, with no death occurring. Based on these results, T-DXd is positioned as standard second-line treatment in patients with metastatic BC, pushing T-DM1 to later treatment options and sparking debate on its role in subsequent lines. Moreover, T-DXd was also evaluated in patients with HER2-low metastatic BC who failed at least one previous line of chemotherapy in the DESTINY-Breast04 phase III trial in comparison to the physician's choice of chemotherapy. The trial met its primary endpoint of PFS in the hormone receptor-positive cohort. The median PFS was 10.1 months in the T-DXd arm *vs.* 5.4 months in the control arm ($P < 0.001$). The median OS was 23.9 months in the T-DXd arm *vs.* 17.5 months in the control group ($P = 0.003$). No new safety concerns were reported^[36]. This trial led to the approval by the FDA of T-DXd for the treatment of adult patients with metastatic HER2-low breast cancer who failed at least one line of chemotherapy. The efficacy of T-DXd in patients with metastatic HER2+ BC with active brain metastases (BMs) has been reported in the TUXEDO-1 phase II trial. T-DXd was associated with an intracranial ORR of 73% (11/15) (95%CI: 48%-89%), including a 13% complete response (CR)^[37]. The DEBBRAH phase II trial also revealed an intracranial ORR of 46.2% in patients with HER2+ metastatic BC with active BMs^[38]. Moreover, subgroup analysis of patients with stable BMs included in the DESTINY-Breast01 and DESTINY-Breast03 trials reported interesting rates of intracranial ORR [58.3 (95%CI: 36.3%-77.9%) and 67.4%, respectively]^[39,40]. [Table 2](#) summarizes major ongoing clinical trials of T-DXd as monotherapy.

MECHANISMS OF RESISTANCE TO ANTI-HER2 ADC

Due to the complexity of the structure and mechanism of action of ADCs, which involve multiple processes, resistance can occur at any stage. It could be related to the expression of an antigen and its identification, or to internalization of the drug and its degradation, or to the release of payload and finally regulation of the apoptosis. To be noted that the large part of data discussed below had been obtained in preclinical models.

Table 2. Major ongoing trials as monotherapy in BC

Study	Agents	Phase	No. of patients	Patients	Primary endpoint
NCT05113251 (DESTINY-Breast11)	ddAC THP vs. T-DXd or T-DXd followed by THP	III	900	High-risk HER2+ early-stage BC	Rate of pCR
NCT05704829 (ADAPTher2-IV)	T-DXd vs. CT + T + P	II	402	Neoadjuvant in low-intermediate risk HER2+ BC	Rate of pCR dDFS
NCT05900206 (ARIADNE)	T-DXd vs. standard of care	II	370	Neoadjuvant treatment HER2+ BC	Rate of pCR
NCT04622319 (DESTINY-Breast05)	T-DXd vs. T-DM1	III	1,600	High-risk HER2+ BC with residual disease in breast or axillary lymph nodes following neoadjuvant therapy	IDFS
NCT04784715 (DESTINY-Breast09)	T-DXd vs. T-DXd + P vs. THP	III	1,157	HER2+ first-line MBC	PFS
NCT04739761 (DESTINY-Breast12)	T-DXd	IIIb/IV	506	Previously treated HER2+ MBC with or without BM	ORR PFS
NCT05950945 (DESTINY-Breast15)	T-DXd	IIIb	250	MBC HER2-low or HER2 IHC 0	TTNT
NCT05744375 (TRANSCENDER)	T-DXd	II	41	First-line treatment MBC resistant to T + P	ORR
NCT06048718 (TUXEDO-4)	T-DXd	II	27	HER2-low MBC with newly diagnosed or progressing BM	ORR

BC: Breast cancer; THP: taxane + Herceptin + pertuzumab; T: trastuzumab; P: pertuzumab; T-DXd: trastuzumab deruxtecan; CT: chemotherapy; pCR: pathological complete response; dDFS: distant disease-free survival; T-DM1: trastuzumab emtansine; IDFS: invasive disease-free survival; MBC: metastatic breast cancer; PFS: progression-free survival; BM: brain metastasis; ORR: objective response rate; TTNT: time to next treatment.

Antigen-related resistance

The most important mechanisms of resistance to HER2-targeting ADCs are related to the exposed antigen. After a long exposure to treatment, the level of expression of the receptor may decrease or structural alterations can occur. After a long treatment with T-DM1 and a high selective pressure, cells showing increased HER2 expression are cleared while those showing decreased HER2 receptor expression are subjected to clonal evolution^[41]. Loganzo *et al.* reported the results of an *in vitro* experience of trastuzumab-maytansinoid (TM-ADC), which is an ADC similar to T-DM1, and BC cells. Two different lines of cells were used: MBA MB-361-DYT2 parental cells, and JIMT1 tumoral cells resistant to first-line treatment with trastuzumab. JIMT1 cells exhibited resistance to TM-ADC and presented responses to other chemotherapies that include anti-tubulin, demonstrating that decreased HER2 expression after months of selective pressure could contribute to drug resistance^[42].

Moreover, the role of tumor heterogeneity in antigen expression and efficacy of ADCs was also reported in the KRISTINE trial. The presence of HER2-negative area in 10% of the cases by FISH or the detection of ERBB2 amplification between 5% and 50% of tumor cells defined HER2 heterogeneity. The pathologic complete response rate was 0% in the 10% of patients presenting tumor heterogeneity^[43]. Furthermore, in the KRISTINE trial, patients with high levels of HER2 heterogeneity before T-DM1 had lower survival outcomes in comparison with those with low heterogeneity^[43]. Similar results were found in the ZEPHIT trial that showed shorter times to treatment failure in patients with high levels of HER2 heterogeneity on HER2-PET CT scans^[44]. Additionally, the antigen ectodomain may present truncated forms of HER2. It was reported as one of the potential resistance mechanisms but has not yet been proven in ADC. It has been initially proven with trastuzumab; tumors with truncated P95HER2 exhibited resistance to trastuzumab, whereas tumors with normal receptors were responsive to trastuzumab^[45]. Heregulin, also known as neuregulin (NRG), which is a ligand for ERBB3 and ERBB4 receptors, could be another potential mechanism that might modulate the sensitivity to ADCs. NRG is a member of the epidermal growth factor (EGF) group, altering

the sensitivity to T-DM1 and stimulating the heterodimerization of HER2 with HER3 and HER4^[46]. Moreover, it seems that HER3 overexpression might be associated with resistance to HER2-targeting drugs such as T-DM1 and trastuzumab^[47]. The inhibition of HER3 reversed acquired resistance to trastuzumab in *in vitro* xenografts derived from HER2-overexpressed gastric cancer^[48].

Payload-related resistance

Another suggested mechanism of resistance to ADC is payload-related resistance that was initially observed in patients with non-Hodgkin lymphomas^[49]. Takegawa *et al.* demonstrated that cells with resistance to T-DM1 had normal overexpression of HER2 but presented upregulated ABCC2 and ABCG2 expression, two ABC transporters that play an important role in chemotherapy resistance. Interestingly, T-DM1 efficacy was restored with the inhibition of these transporters, suggesting that amplified DM1 efflux contributed to resistance to T-DM1^[50].

Intrinsic mechanisms of resistance to ADCs

One of the intrinsic mechanisms of resistance is the failure of internalization of HER2 by endocytosis. Numerous pathways of internalization were reported, such as clathrin-mediated endocytosis mediated by clathrin, the most common way of endocytosis of ADCs, endocytosis mediated by caveolae, and endocytosis independent of clathrin-caveolin^[51]. Sung *et al.* generated multiple *in-vitro* models of resistance to T-DM1^[52]. The aforementioned mechanism (reduced HER2 expression) was found in two models. However, N87-TM model had normal transporters' expressions and the same level of HER2 expression. N87-TM resistant cells to T-DM1 internalized HER2 receptor via caveolin-1 (CAV-1)-coated vesicles. In fact, the overexpression of CAV-1 was negatively correlated with HER2 expression over cell membranes and was associated with decreased colocalization of lysosomes and reduced T-DM1 sensitivity. Preclinical data demonstrated that colocalization of CAV-1 and T-DM1 was associated with lower sensitivity to T-DM1, suggesting CAV-1 as a predictive biomarker of T-DM1 resistance and other types of ADCs with non-cleavable linkers^[52-55]. Moreover, endophilin A2, a protein involved in clathrin-independent endocytosis and encoded by SH3GL1, increases HER2 internalization in patients with HER2-positive BC and is associated with a higher response of tumor cells to T-DM1 and trastuzumab. It has also been demonstrated that the inhibition of SH3GL1 in cancer cells reduced the internalization and contributed to lower cytotoxicity mediated by T-DM1^[56].

After the internalization of ADCs and their target molecules via endocytosis, they are driven to lysosomes, and cytotoxic agents are released after chemical and enzymatic cleavage. An impaired lysosomal function can contribute to resistance to ADCs. In fact, Ríos-Luci *et al.* isolated three resistant HER2-positive clones following treatment with T-DM1. These clones had the same expression of HER2, normal internalization and trafficking pathways. However, they presented increased pH levels in the lysosomes, along with altered proteolysis, resulting in the accumulation of T-DM1 and subsequently limiting its antitumor activity^[57]. Moreover, defective liberation of antineoplastic drugs from lysozymes or defective traffic to cytoplasm might be implicated in the resistance to ADCs. *In vitro* models showed that the silencing or loss of SLC46A3, a gene encoding for a protein that is a direct transporter of maytansine-based catabolites outside the lysosomes, led to the accumulation of catabolites in the lysosomes and subsequent acquired T-DM1 resistance^[58]. Regarding T-DXd, the DAISY multicenter phase II trial was the first study investigating the mechanisms of action of T-DXd as well as the mechanism of resistance that was unclear. The DAISY trial enrolled patients with metastatic BC into three cohorts: HER2-positive cohort ($n = 72$ patients), HER2-low ($n = 74$ patients), and HER2-zero ($n = 40$ patients). T-DXd was given at the dosage of 5.4 mg/kg every three weeks until progression or unacceptable toxicity. The primary endpoint of the study was the best objective response. Interestingly, tumor samples were required before the beginning of the treatment and at progression. In addition, whole exome sequencing was performed on these biopsies. It has been demonstrated that T-DXd distribution was lower in the HER2-zero cohort in comparison with the HER2-positive cohort ($P = 0.05$).

Additionally, enrichment of serotonin and G-protein coupled receptor signaling was found in the HER2-positive cohort after T-DXd treatment. The WES found that mutations in SLX4, a gene that plays an important role in regulating endonucleases and DNA damage repair, were present in 20% of patients. The DAISY trial found that depletion of SLX4 led to cancer cell resistance to treatment. Moreover, loss-of-function mutations could be identified as a potential mechanism of resistance to treatment^[59].

Drug efflux pumps were also reported as a mechanism of resistance to treatment by enhancing treatment ejection from tumor cells and could contribute to the occurrence of cancers with multidrug-resistant (MDR) phenotypes^[60]. It could also be considered as a mechanism of resistance to ADC. The most frequent phenotype is the MDR1-mediated efflux. For example, maytansinoids are substrates of ABC transporters such as MDR1^[61]. Moreover, *in vitro* data showed that the inhibition of MDR1 led to the reversal of the resistance to T-DM1^[62].

Signaling pathways activation

Another potential mechanism that might contribute to resistance to ADCs is the activation of signaling pathways. The most common pathway involved is the PI3k/AKT/mTOR pathway, which is responsible for tumor growth, metabolism, and survival. It has been reported that the activation of the PI3K pathway can contribute to reduced response to ADCs, decreased activity of the cytotoxic drug, and longer survival of the cells in patients treated with trastuzumab who have PTEN deletions or PIK3CA mutations^[63]. To date, available data from the EMILIA trial show that patients with PIK3CA mutation or PTEN deletion and receiving T-DM1 had the same survival outcomes compared to those without genetic alterations^[64]. Moreover, Wu *et al.* reported that the overexpression of Wnt3 favors higher expression of β -catenin, increased cell proliferation rate, cell invasiveness and resistance to trastuzumab, indicating that the activation of the Wnt/ β -catenin pathway might be involved in resistance to ADCs^[65]. Another potential mechanism of resistance is the overexpression of the trophoblast cell-surface antigen 2 (TROP-2), which is a transmembrane glycoprotein that could be implicated in the akt phosphorylation in different tumoral cells, and in the activation of several signaling pathways such as PI3K/Akt, MAPK/ERK, and Wnt/ β -catenin pathways^[66]. Recently, Gion *et al.* reported a retrospective analysis from the PHERGain trial of the relationship between TROP-2 expression and pathological complete response (pCR). Patients with TROP-2 expression had significantly lower pCR ($P = 0.01$). These findings suggest that TROP-2 could be a potential biomarker of resistance^[67]. Moreover, the Topoisomerase II Alpha (TOP2A) gene is located at chromosome 17 and near the HER2/neu gene. It encodes for topoisomerase II alpha protein. Interestingly, it has been reported that 35% of patients with BC harboring HER2 amplification present with co-amplification of the TOP2A gene^[68]. The topoisomerase II alpha protein is the target of anthracyclines, notably doxorubicin and epirubicin^[69]. The role of TOP2A in the resistance to anti-HER2 targeted therapies is unclear. However, it could be intriguing to develop agents that target HER2 and TOP2A.

OVERCOMING RESISTANCE TO ADC

Multiple strategies are currently under evaluation in order to prevent or reverse resistance to anti-HER2 ADCs that remains a significant challenge, and to ameliorate their clinical activity in metastatic BC. These strategies might be the use of other ADCs, more specifically, next-generation ADCs with different target or payload or DAR, or combination with other agents such as tyrosine kinase inhibitors (TKIs), immune checkpoint inhibitors (ICIs), and synthetic DNA-damaging agents. Tables 3 and 4 summarize major ongoing clinical trials evaluating T-DM1 and T-DXd combined with other drugs. Moreover, Durbin *et al.* reported in preclinical models that the reduction of antigen/antibody complex recycling to the membrane could be a possible target for pharmacological intervention^[70].

Table 3. Ongoing studies evaluating T-DM1 combined with other drugs in BC

Trial	Agents	Phase	Number of patients	Patients	Primary endpoint
NCT05673928 (TUCATEMEB)	Tucatinib in combination with T-DM1	II	30	Metastatic HER2-positive cancers	Intracranial antitumor activity
NCT04873362 (Astefania)	T-DM1 + placebo or atezolizumab	III	1,700	HER2-positive BC with high risk of relapse in the adjuvant setting	IDFS
NCT04457596 (CompassHER2 RD)	T-DM1 + placebo or tucatinib	III	1,031	HER2-positive BC with residual disease following neoadjuvant HER2-based treatment	IDFS
NCT05560308	T-DM1 + pyrotinib maleate	II	50	HER2+ MBC after progression on TKI	ORR

T-DM1: Trastuzumab emtansine; BC: breast cancer; IDFS: invasive disease-free survival; MBC: metastatic breast cancer; TKI: tyrosine kinase inhibitor; ORR: overall response rate.

Table 4. Ongoing studies evaluating T-DXd associated with other agents in BC

Study	Agents	Phase	Number of patients	Patients	Primary endpoint
NCT04539938 (HER2CLIMB-04)	Tucatinib + T-DXd	II	70	HER2-positive MBC progressing on trastuzumab + taxanes	ORR
NCT05633979	T-DXd + valemetostat	Ib	37	HER2-low/O MBC	Safety MTD ORR RDE
NCT05795101 (TRUDI)	T-DXd + durvalumab	II	63	Previously untreated with HER2+/low Inflammatory BC	pCR
NCT05372614	T-DXd + neratinib	I	18	Metastatic HER2-altered tumors	DLTs TEAEs
NCT04538742 (DESTINY-Breast07)	T-DXd + durvalumab or tucatinib or pertuzumab or paclitaxel	I/II	245	Previously untreated HER2-positive MBC	AEs SAEs
NCT04042701	pembrolizumab + T-DXd	I	115	HER2-positive tumors after progression on T-DM1 or HER2-low MBC	DLTs ORR
NCT04585958	Olaparib + T-DXd	I	55	Metastatic HER2-altered tumors	MTD, RP2D AEs

T-DXd: Trastuzumab deruxtecan; BC: breast cancer; MBC: metastatic breast cancer; ORR: objective response rate; MTD: maximum tolerated dose; RDE: recommended dose for expansion; pCR: pathologic complete response; DLTs: dose-limiting toxicities; TEAEs: treatment-emergent adverse events; AEs: adverse events; SAEs: serious adverse events; T-DM1: trastuzumab emtansine; RP2D: recommended phase 2 dose; AEs: adverse events.

Next-generation ADCs

Regarding next-generation ADCs, the previously discussed DESTINY-Breast02 phase III trial is the first randomized study proving that an ADC (T-DXd) can overcome the resistance to another one (T-DM1)^[35].

Trastuzumab duocarmazine, formerly named SYD985, is a HER2-targeting monoclonal antibody coupled to a duocarmycin (alkylating agent) via a C linker. The DAR of SYD985 is 2.6. It has been investigated in a phase I study enrolling patients with locally advanced or metastatic solid tumors with HER2 immunohistochemistry of 1+ or more. The study included patients with heavily pretreated metastatic BC. The dose-limiting toxicity was 2.4 mg/kg (related to pneumonitis) and the recommended phase 2 dose was 1.2 mg/kg. In the dose-expansion cohort, the ORR was 33% and 32% in patients with HER2-positive and HER2-low metastatic BC, respectively. Regarding safety profile, grade 3 or more AEs were observed in 35% of participants^[71]. The TULIP phase III trial randomized patients with HER2-positive metastatic BC who

failed at least two previous lines of treatment to either SYD985 or treatment of physician's choice. Previous treatment with T-DM1 was allowed. The study met its primary endpoint of PFS. SYD985 significantly prolonged PFS in comparison with control group (7.0 vs. 4.9 months, HR: 0.64; $P = 0.002$). However, the final analysis of OS did not show a survival advantage with SYD985 (HR: 0.87; 95% CI, 0.68-1.12; $P = 0.236$) and both arms showed similar ORR. The rate of interstitial lung disease/pneumonitis was 7.6% (22/288 patients), including two grade 5 events (grade 1-2 in 5.2% of patients). To be noted that 21.2% of patients presented grade 3 or more ocular toxicities in the SYD985 group that led to treatment withdrawal in 20.8% of patients^[72]. The ISPY-P1.01 is a phase I trial evaluating SYD985 in combination with paclitaxel in metastatic BC (NCT04602117).

Trastuzumab rezetecan (SHR-A1811) is a new-generation HER2-directed ADC composed of trastuzumab linked to a novel topoisomerase I inhibitor payload (SHR9265) via a stable and cleavable linker. It has been studied in a phase I first-in-human study including pretreated patients with HER2-mutated/expressing advanced solid tumors. The entire cohort has an ORR of 61.6% (154/250). Interestingly, patients with HER2-positive BC had an ORR of 81.5% (88/108 patients), while patients with HER2-low disease had an ORR of 55.8% (43/77). Moreover, patients with HER2-positive BC and previously treated with T-DM1 had an ORR of 82.4% (14/17). Fifteen patients had previously received an anti-HER2 ADC other than T-DM1, including T-DXd. The ORR was 60% in these patients (9/15)^[73]. A166 is another HER2-directed ADC composed of humanized HER2-targeting antibody linked to a cytotoxic drug (Duostatin-5, a microtubule inhibitor agent) via a stable cleavable linker. In the cohort of HER2-positive patients, A166 was associated with an ORR of 70%. In addition, median PFS ranged between 9.4 and 12.3 months following the administered doses^[74].

Disitamab vedotin (RC48) is a humanized anti-HER2 ADC conjugated with monomethyl auristatin E (MMAE) via a cleavable linker with a bystander effect on cancer cells. The DAR for RC48 is 4.1. In a preclinical model of HER2-positive BC metastatic to the lungs, tumor cells showed resistance to T-DM1 and T-DXd and were moderately sensitive to disitamab vedotin. In the mouse model, the three drugs inhibited the development of lung metastases, and T-DXd and disitamab vedotin were more efficacious than T-DM1^[75]. Recently, pooled analysis from two phase I trials in patients with HER2-positive and HER2-low metastatic BC demonstrated clinical activity of RC48. The ORR was 40% in the HER2-low group and 43% in the HER2-positive group. The RP2D was 2 mg/kg. The drug presented an acceptable safety profile: decreased neutrophil count, fatigue, and increased GGT were the most frequent grade 3 or more AEs^[76]. Several phase III trials are currently investigating RC48 in patients with locally advanced or metastatic HER2-positive or HER2-low BC (NCT03500380 and NCT04400695, respectively).

XMT-1522 is another novel HER2-targeting ADC and was compared to T-DM1 *in vitro* and *in vivo* in gastric cancer and BC. Le Joncour *et al.* found that XMT-1522 showed efficacy in BC and gastric cancer cells expressing HER2 and xenograft models both resistant to T-DM1^[77]. Moreover, ARX788 is an anti-HER2 ADC consisting of anti-HER2 mAb conjugated to amebastatin (AS269) cytotoxic payload. *In vitro* and *in vivo* experience showed that ARX788 is efficacious in models of breast and gastric cancers expressing HER2, including models with acquired resistance to T-DM1^[78,79]. In the phase I trial, ARX788 showed activity in 31% of patients with heavily pretreated patients with HER2-positive metastatic BC^[80]. In the ACE-BREAST-02 phase III trial, ARX788 was compared with capecitabine and lapatinib in patients who were previously treated with trastuzumab and taxane. The study met its primary endpoint of PFS^[81].

Bispecific or biparatopic mAbs are a promising solution to overcome or prevent resistance to ADCs. Li *et al.* engineered *in vitro* models of HER2-targeting bivalent biparatopic ADCs that demonstrated efficacy against T-DM1 resistant tumors as well as HER2-low expressing cancers. The benefit is due to the targeting of two

non-overlapping epitopes on HER2 inducing HER2 receptor clustering and consequently robust internalization, lysosomal trafficking, and degradation^[82]. Moreover, Zanidatamab zodovotin (ZW49) is a bispecific ADC targeting two non-overlapping HER2 antigens at the same time and linked to an auristatin payload via a C linker. Its DAR is 2. It has been evaluated in HER2-positive solid tumors in a phase I dose-escalation trial. Two dose-limiting toxicities have been reported at the dosage of 1.75 and 2.5 mg/kg. It should be noted that treatment-related keratitis was observed in 43% of patients. The DCR in the cohort of 2.5 mg/kg was 50% in patients with HER2-positive metastatic BC^[83]. BIO-201 is a novel bispecific ADC that targets TROP-2 and HER2 conjugated with a topoisomerase I inhibitor via a cleavable linker. Preclinical data suggest that BIO-201 is effective in different types of cancer co-expressing TROP-2/HER2 or either of the targets^[84].

As previously mentioned, HER3 overexpression can lead to resistance to anti-HER2 drugs. HER3 inhibition using HER3-targeting ADC such as patritumab deruxtecan could be an interesting area to investigate. Patritumab deruxtecan (HER3-DXd) is a HER3-targeting ADC coupled to TOPO1 inhibitor via a C linker. The DAR is 8. It has been evaluated in the U31402-A-J101 phase I/II trial in patients with HER3-expressing metastatic BC. Interestingly, in the subgroup of patients with HER2-positive BC ($n = 14$), HER3-DXd was associated with remarkable clinical activity with ORR of 42.9%, DCR of 92.9%, and median PFS of 11.0 months^[85]. Zenocutuzumab is a bispecific antibody targeting HER2 and HER3 simultaneously. It has been evaluated in patients with cancers harboring NRG1 fusion. In a phase II trial, responses to zenocutuzumab were observed in 34% of patients (35% in patients with NSCLC, 39% in patients with pancreatic adenocarcinoma, and 50% in patients with BC)^[86].

Another strategy to overcome resistance to ADC and increase sensitivity to these agents is the development of non-internalizing ADCs that could target the tumor microenvironment (TME). In fact, the TME, mainly composed of extracellular matrix, abnormal blood vessels and stromal cells such as cancer-associated fibroblasts, plays a crucial role in the growth of tumor cells, neoangiogenesis, and the removal of metabolic waste^[87]. The TME is associated with some antigens that include enzymes, transcription factors, and checkpoint molecules^[88]. Several novel ADCs are under clinical development and evaluation in hematological malignancies and solid tumors targeting CD74, CCR7, and CD276^[89,90].

Combination strategies with ADC

Multiple combinations are currently under investigation to overcome acquired resistance to HER2-targeting ADCs and improve the outcomes after failure of these drugs. The most evaluated and promising associations are ADCs with TKI, ICIs, and DNA-damaging agents such as PolyADP-Ribose Polymerase-1 (PARP) inhibitors. The HER2CLIMB-02 is the first phase III study to compare tucatinib combined with T-DM1 vs. T-DM1 alone in patients with unresectable HER2-positive BC who received a combination of taxanes with trastuzumab. The results were presented at the 2023 San Antonio Breast Cancer Symposium, and the trial met its primary endpoint of PFS. The combination significantly prolonged PFS in comparison with T-DM1 alone (9.5 vs. 7.4 months, HR 0.74). To be noted that 44.1% of patients enrolled in the study had brain metastases at baseline^[91]. The major limitation of this trial is that it does not compare the combination with T-DXd that is currently the standard of care as a second-line treatment. Moreover, the positioning of this combination is not very clear. Although the results of trials should not be compared, T-DXd was associated with a median PFS of 28.8 months in second line, 17.8 months in T-DM1 refractory patients vs. 9.5 months in the HER2CLIMB02. The HER2CLIMB-04 is an ongoing phase II trial evaluating tucatinib in combination with T-DXd in patients with HER2-positive metastatic BC (NCT04539938).

Regarding the combination with ICIs, the KATE2 phase II randomized trial was the first study to evaluate the combination of T-DM1 with atezolizumab, a programmed death ligand-1 (PD-L1), in patients with previously treated HER2-positive metastatic BC. The trial did not meet its primary endpoint of PFS in the

intention-to-treat population (8.2 vs. 6.8 months; HR: 0.82, 95%CI: 0.55-1.23; $P = 0.33$), showing that the addition of atezolizumab to T-DM1 did not improve PFS and was associated with more adverse events^[92]. The subsequent KATE3, randomized phase III trial is comparing T-DM1 in combination with atezolizumab vs. T-DM1 alone in patients with previously treated HER2-positive and PD-L1-positive metastatic BC (NCT04740918)^[93]. Moreover, the ASTEFANIA phase III trial is also evaluating T-DM1 and atezolizumab or placebo in patients with HER2-positive BC at a high risk of recurrence following neoadjuvant chemotherapy (NCT04873362)^[94]. The combination of T-DXd with nivolumab has also been evaluated in a phase 1b trial in patients with HER2-positive or HER2-low metastatic BC. In the HER2-positive metastatic BC, the combination was associated with antitumor activity consistent with prior results of T-DXd alone, raising the question of the utility of adding ICIs to ADCs, which requires more follow-up and additional studies^[95].

CONCLUSION

As previously mentioned, the HER2-targeting landscape has dramatically changed the treatment landscape of patients with HER2-positive BC as well as HER2-low metastatic BC. To date, two drugs are approved by the FDA and EMA for patients with HER2-expressing breast cancer. T-DM1 is the first approved ADC and is currently indicated as adjuvant treatment in patients with HER2-positive BC with residual disease following neo-adjuvant trastuzumab-based chemotherapy. T-DXd is the second HER2-targeting approved ADC in HER2-positive and HER2-low metastatic BC. It defeated T-DM1 in a head-to-head comparison in second-line settings in patients with HER2-positive metastatic BC and sparked the debate on the efficacy of T-DM1 as monotherapy in subsequent therapeutic lines. The impressive clinical efficacy of T-DXd is counterbalanced by the emergence of resistance to this agent and the absence of clinical data and standardized consensus for the treatment of patients after progression on T-DXd. The preferred available regimens could be T-DM1 or the combination of capecitabine, tucatinib, and trastuzumab following the results of the HER2CLIMB phase III trial that showed statistically longer median OS in comparison with capecitabine and trastuzumab^[96]. The combination of T-DM1 and tucatinib could be another option. Moreover, ongoing clinical trials are assessing T-DXd as a potential first-line treatment, comparing it with the current standard of care and exploring its use in the adjuvant setting. If approved for these indications, it will further complicate the treatment landscape for patients with HER2-positive breast cancer. Investigating next-generation ADCs is crucial, with a need for more efforts to elucidate their mechanisms of action, pharmacodynamic and pharmacokinetic profiles, and the molecular drivers of resistance, which remain poorly understood. Another pertinent concern is whether an ADC can effectively reverse the resistance to another ADC. While this has been demonstrated with T-DXd following T-DM1, it requires confirmation with next-generation ADCs. Combination strategies also offer promise in overcoming resistance, supported by encouraging available data.

DECLARATIONS

Authors' contributions

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The epigenetic mechanisms involved in the treatment resistance of glioblastoma

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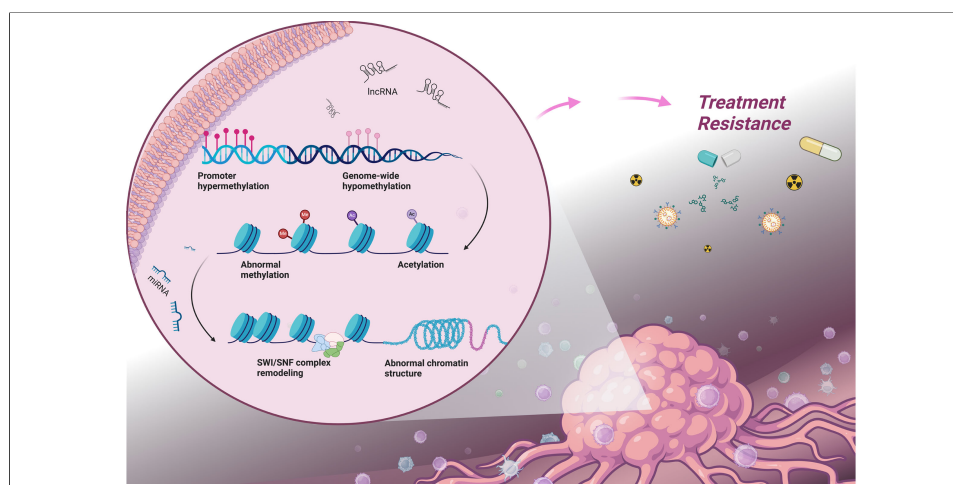
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Abstract

Glioblastoma (GBM) is an aggressive malignant brain tumor with almost inevitable recurrence despite multimodal management with surgical resection and radio-chemotherapy. While several genetic, proteomic, cellular, and anatomic factors interplay to drive recurrence and promote treatment resistance, the epigenetic component remains among the most versatile and heterogeneous of these factors. Herein, the epigenetic landscape of GBM refers to a myriad of modifications and processes that can alter gene expression without altering the genetic code of cancer cells. These processes encompass DNA methylation, histone modification, chromatin remodeling, and non-coding RNA molecules, all of which have been found to be implicated in augmenting the tumor's aggressive behavior and driving its resistance to therapeutics. This review aims to delve into the underlying interactions that mediate this role for each of these epigenetic components. Further, it discusses the two-way relationship between epigenetic modifications and tumor heterogeneity and plasticity, which are crucial to effectively treat GBM. Finally, we build on the previous characterization of epigenetic modifications and interactions to explore specific targets that have been investigated for the development of promising therapeutic agents.



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INTRODUCTION

The most common primary malignant brain tumor is glioblastoma (GBM). Approximately, around 13,000 individuals are diagnosed with GBM every year in the United States^[1]. GBM is characterized by its aggressive nature and ability to recur. In this context, the 5-year survival rate of patients remains below 7% despite multidisciplinary management with surgical resection, radiation therapy, and chemotherapy^[1]. Hence, the tumor's resistance to treatment remains a major concern and a topic of substantial research interest. This resistance is a multifaceted process with several underlying mechanisms. One of the major contributors to the development and maintenance of treatment resistance is the epigenetic profile of GBM cells, which is defined as the set of alterations and effectors that regulate the expression of genes and the resultant phenotype of cells without changing their DNA sequence^[2,3]. These epigenetic processes include DNA methylation and demethylation, histone modifications, chromatin remodeling, and non-coding RNAs. Each of these modifications, along with the enzymes and molecular pathways that interact with them, have been shown to be implicated in driving treatment resistance in at least one mechanism. This review aims to highlight the significant implication of epigenetic alterations and processes in GBM's resistance to therapeutic modalities. It also discusses the heterogeneity and plasticity present within GBM tumors, which include the diverse epigenetic profiles. Finally, the review builds on these aspects to explore targeted agents and therapeutic modalities that have been shown to interfere with the epigenetic profile of GBM tumors and recircuits it in a way that attenuates treatment resistance, augments the efficacy of existing therapeutics, and potentially could improve patient outcomes.

EPIGENETIC PROCESSES IMPACT GLIOBLASTOMA RESISTANCE TO TREATMENT

DNA methylation

DNA methylation, initially documented in 1948, stands as one of the most extensively researched epigenetic modifications. In humans, this process involves the attachment of a methyl group to cytosine residues, primarily at CpG sites, facilitated by a group of enzymes known as DNA methyltransferases (DNMTs). One-carbon metabolism provides the methyl groups necessary for the methylation of cytosine residues in DNA, particularly in CpG islands, influencing gene expression and chromatin structure. Analysis of glioblastoma surgical specimens from both initial presentation and recurrence shows that enzymes involved in one-carbon (1-C) purine synthesis are upregulated in recurrent glioblastoma. Furthermore, higher expression of these enzymes is associated with a shorter time to tumor recurrence^[4]. DNA methylation serves several pivotal roles across various stages of human development and throughout life, including transcriptional regulation, genomic imprinting, preservation of X-chromosome inactivation, chromosomal maintenance, and upkeeping genomic stability^[5-7]. Aberrant DNA methylation, observed in cancer cells, involves genome-wide hypomethylation and site-specific hypermethylation, primarily affecting CpG islands located within gene expression regulatory regions. These alterations play a significant role in tumor initiation, advancement, and resistance to treatment^[8]. In addition to the more conventionally explored CpG island methylation, another form of non-CpG DNA methylation has been investigated. This form of methylation is known as CpH (where H refers to any base other than G). Herein, CpH methylation especially occurs in the brain^[9] and has been associated with alpha-synuclein expression in Parkinson's disease^[10] and with risk loci in Schizophrenia^[11]. However, to date, the impact of CpH methylation has not been explored in the context of brain tumors and its potential association with oncogenic gene expression and aggressive phenotypes in these tumors.

In the context of GBM, DNA methylation frequently targets the promoter regions of tumor suppressor genes, which are responsible for restraining cell growth, division, and promoting programmed cell death. When these genes undergo silencing via DNA methylation, their ability to suppress tumor growth is compromised, leading to uncontrolled proliferation of tumor cells. Examples of tumor suppressor genes affected by DNA methylation in GBM include *Phosphatase and tensin homolog (PTEN)* and

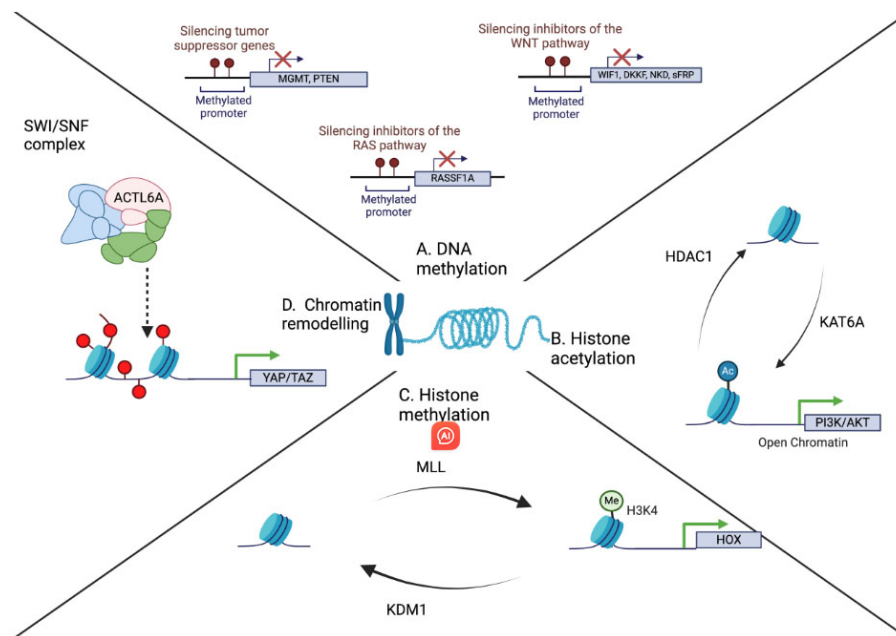


Figure 1. Illustration of the different epigenetic modifications that target chromatin and contribute to treatment resistance in GBM. (A) DNA methylation silences the promoters of tumor suppressor genes, hence contributing to the proliferative ability of GBM cells. This mechanism also has the ability to suppress the expression of inhibitors of the WNT pathway (WIF1, DKKF, NKD, sFRP) and RAS pathway (RASSF1A); (B) Histone acetylation, which is achieved by histone acetylases and reversed by histone deacetylases, can also contribute to the regulation of chromatin structure and gene expression. For example, histone acetylation by KAT6A can lead to increased expression of the genes involved in the overactivation of the PI3K/AKT oncogenic pathway. This is reversed by the histone deacetylase HDAC1; (C) Histone methylation is another alteration that can control the expression of genes. For instance, the interplay between the histone methylase MLL and the demethylase KDM1 can regulate the expression of HOX genes, which are implicated in cancer proliferation and treatment resistance; (D) The chromatin remodeling complex SWI/SNF can alter the architecture of chromatin through several of its domains. One such domain, ACTL6A, can promote the expression of the YAP/TAZ pathway, which, in turn, contributes to treatment resistance. GBM: Glioblastoma multiforme; MGMT: methylguanine methyltransferase; PTEN: phosphatase and tensin homolog; WNT: wingless; WIF: WNT inhibitory factor; DKKF: dickkopf; NKD: naked cuticle; sFRP: secreted frizzled-related protein family; RASSF1: ras association domain family; HDAC: histone deacetylase; KAT: lysine acyltransferase; MLL: mixed lineage leukemia; KDM: histone lysine demethylase; SWI/SNF: switch/sucrose non-fermentable; ACTL6A: actin-like protein 6A; PI3K/AKT: phosphoinositide-3-kinase-protein kinase B; HOX: homeobox; YAP/TAZ: yes-associated protein/transcriptional co-activator with PDZ-binding motif.

*O*6-methylguanine-DNA methyltransferase (MGMT)^[12,13] [Figure 1A]. In GBM, this DNA methylation disrupts transcription factors binding to the promoter region, thus inhibiting the initiation of gene expression^[14].

The *MGMT* gene encodes the DNA-repair protein *O*6-alkylguanine-DNA-alkyltransferase (AGT)^[15]. AGT plays a crucial role in cellular physiology by removing alkylating lesions at the *O*6 position of guanine, thereby maintaining genomic stability and preventing DNA damage, including that induced by alkylating chemotherapeutic agents such as temozolomide (TMZ)^[16]. *MGMT* expression varies widely among different types of tumors and normal tissues. Higher levels of *MGMT* expression contribute to increased DNA stability and protect cells from the deleterious effects of alkylating agents used in chemotherapy^[15]. Conversely, decreased or absent *MGMT* expression increases the susceptibility to chemotherapeutic agents and enhances sensitivity to alkylating agents^[15]. In tumors, *MGMT* promoter methylation effectively silences *MGMT* expression, leading to increased responsiveness to chemotherapy that includes alkylating agents^[17]. In the clinical setting, in GBM, *MGMT* promoter methylation status helps predict the efficacy of TMZ therapy, since patients with *MGMT* promoter methylated tumors tend to have a better response to TMZ chemotherapy and longer overall survival compared to those without promoter methylation^[18,19].

Moreover, DNA methylation can impact genes involved in the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway, which is frequently dysregulated in GBM, altering the sensitivity of tumor cells to targeted therapies [Figure 1B]. In general, the phosphoinositide-3-kinase-protein kinase B (PI3K-AKT) signaling pathway promotes cellular proliferation and growth while concurrently suppressing apoptosis^[20]. Singh *et al.* demonstrated that depletion of TP53, *PTEN*, and NF1 in human brain organoids induces a glioma-like phenotype *in vitro*, underscoring the significance of *PTEN* suppression in gliomas^[21]. Wiencke *et al.* showed that methylation of the *PTEN* promoter is associated with phosphorylation of protein kinase B (PKB/Akt), indicating functional activation of the PI3K pathway^[22]. This activation contributes to the promotion of GBM growth and enhancement of cell survival.

Hes Related Family BHLH Transcription Factor with YRPW Motif 1 (HEY1) belongs to the Hairy/Enhancer of split [H/E(spl)] family of basic helix-loop-helix transcription factors^[23]. It plays a crucial role in sustaining neural precursor cells following Notch signaling. The Notch receptor undergoes cleavage by TNF- α -converting enzyme and γ -secretase, resulting in the generation of the active Notch intracellular domain (NICD)^[24]. NICD translocates to the nucleus, where it facilitates the expression of Notch targets such as HEY1 and hairy and enhancer of split-1 (HES1)^[25]. Notch signaling is essential in GBM. Notch receptors and their ligands are markedly upregulated in GBM, indicating abnormal activation of Notch signaling^[26]. Elevated levels of Notch1 and NICD1 are frequently observed in GBM. Increased activation of Notch1-mediated signaling contributes to the development and resistance to chemotherapy in GBM^[24,27-29]. Hence, targeting the Notch pathway has emerged as a promising approach for future GBM therapies^[24]. HEY1 expression rises proportionally with the severity of astrocytoma tumor grades and is associated with both reduced overall survival and reduced disease-free survival rates^[23]. Studies, such as that by Tsung *et al.*, have shown that the methylation status of HEY1 plays a role in GBM pathogenesis and can serve as a predictive marker for GBM patients^[30].

In GBM, aberrant promoter methylation contributes to the activation of the Wingless (WNT) pathway by silencing various negative regulators. These include genes encoding WNT inhibitory factor 1 (WIF1), members of the secreted frizzled-related protein (sFRP) family, Dickkopf (DKKF), and naked cuticle (NKD) family members^[31,32]. Likewise, promoter methylation also silences negative regulators of the Ras pathway, such as the Ras association (*RalGDS/AF-6*) domain family member *RASSF1A*^[33] [Figure 1].

Histone modification

The role of histone post-translational modifications (PTMs) in regulating gene expression is well recognized^[34]. This is primarily accomplished by altering the structure and/or function of chromatin, an octamer core consisting of paired copies of histone proteins H2A, H2B, H3, and H4 enveloped by double-stranded DNA^[35]. Attached to these histones are N-terminal tails, which extend beyond the nucleosome core and are thus susceptible to PTMs, including methylation, acetylation, phosphorylation, and ubiquitination. Abnormalities within these histone modifications have been demonstrated to contribute to gene transcription, resulting in increased GBM proliferation, invasion, and ultimately therapeutic resistance^[36-39].

Histone methylation and demethylation

Methylation of histone tails occurs because of the actions of histone methyltransferases (HMTs), which catalyze the transfer of methyl groups from S-adenosylmethionine onto the fundamental residues of a histone tail^[40]. Methylation most commonly occurs on the side-chain nitrogen atoms of arginines and lysines^[41] [Figure 1C]. Accordingly, HMTs are generally divided into lysine methylating proteins (KMTs), such as SET [Su(var), Enhancer of Zeste, and Trithorax] domain-containing and DOT1-like proteins, and

arginine methylating proteins such as the arginine N-methyltransferases (PRMTs)^[41]. Unlike acetylation, methylation alters the hydrophobicity and hydrogen binding radii of methyl-lysine, thereby modifying the binding properties of these sites without neutralizing the charge of the target residue^[42]. Reversal of histone methylation is catalyzed by histone demethylases. Similar to their methylating counterparts, demethylases are segregated between lysine demethylation enzymes (KDMs), such as amine oxidase domain-containing (LSD) proteins and Jumonji C (JmjC) domain-containing proteins, while identifying arginine-selective demethylases has proven generally elusive^[43,44].

In humans, histone H3 is arguably the most cited due to its highly conserved sequence in eukaryotic organisms, which enables the identification of numerous PTMs on the histone^[45]. Particularly, methylation occurs on the lysine residue sites of H3, with H3K4, H3K36, and H3K79 methylations marking transcriptionally active genes and H3K9, H4K20, and H3K27 methylations signaling inactive genes^[46-48]. For example, it is understood that H3K4 is methylated by the mixed lineage leukemia (MLL) family of proteins in GBM biology-leading to upregulation of genes implicated in differentiation and self-renewal [Figure 1C]^[46,49]. Furthermore, MLL has been shown to directly increase the GBM stem-like cell (GSC) proliferation rate *in vitro* and *in vivo* through activation of the HOXA10 transcription factor and subsequent upregulation of developmental Hox genes^[50]. Another example is enhancer of Zeste homolog 2 (EZH2), the primary HMT enzyme for H3K27me methylation, which has been increasingly aligned with GBM tumorigenicity, stemness, and resistance to therapeutics such as TMZ through its upregulation of c-MYC expression and STAT3 phosphorylation^[51-57]. Moreover, Su(var)3-9/enhancer-of-zeste/trithorax (SET) domain and mariner transposase fusion gene (SEMTAR), an additional HMT responsible for H3K36 methylation, has been highlighted as a contributor to GBM radiation resistance through its recruitment of Ku80, a DNA damage repair protein^[57]. Although mechanistic pathways for HMT influence are generally unclear, it is evident that they present unique targets for potential epigenetic treatment avenues. Similarly important to GBM tumorigenicity is the H3K9 family of methyltransferases. Euchromatic histone lysine methyltransferase 2 (EHMT2) expression has been linked to a pro-tumorigenic effect^[58,59], with *in vitro* inhibition reducing overall H3K9 dimethylation and increasing c-MYC-dependent autophagy and autophagy-dependent differentiation. Additionally, EHMT2 methylation of hypoxia-inducible factor-1 (HIF-1) inhibited hypoxia adaptation and cellular invasion in the U251MG cell line, ultimately revealing a hypoxia-induced mechanism of negative feedback for HIF-1 activity and human GBM cell mobility^[60,61]. Similarly, methylation of H3K9 by Suv39H1 and SETDB1 has demonstrated decreased expression of the gene. Further studies in GBM have noted an increased expression of both Suv39H1 and SETDB1 as compared to normal brains, with short-hairpin-RNA-mediated (shRNA) knockdown of SETDB1 or chaetocin-mediated inhibition of Suv39H1 being associated with increased apoptosis and reduced migration and colony formation in T98G and GOS-3 glioma cell lines^[62-65].

Generally, however, histone lysine methylation serves to modify the regulation of transcription and chromatin structure dependent on the degree and site of methylation. Notably, this includes the association of H3K4 monomethylation with enhancer regions, di- and trimethylation of H3K4 with promoter regions and transcription start sites, and trimethylation of H3K36 in gene bodies of actively transcribed genes^[66]. A more in-depth analysis of biological histone lysine methyltransferases has been published by Husmann and Gozani^[67]. Counteracting these methylating efforts are KDMs such as KDM1, which demethylates H3K4 and H3K9, KDM2, which demethylates H3K36, and KDM4, which demethylates H3K9. In adult and pediatric GBM, the KDM4 subfamily of lysine demethylases retains a significant functional impact, with studies demonstrating *in vitro* elevation of KDM4A expression in the more TMZ-resistant T98G cell lines as compared to the U251MG^[68]. Additionally, KDM2A plays an integral role in GBM immune resistance through a miR-302a/KDM2A/JAG1 axis, resulting in increased T-regulatory cell proliferation in mouse models^[69,70]. Moreover, research by Liao *et al.* demonstrated that GSCs can enter a slow-cycling or quiescent

state in response to receptor tyrosine kinase (RTK) inhibition. These slow-cycling cells depend on histone demethylase KDM6A/B, which leads to the redistribution of histone H3 lysine 27 trimethylation (H3K27me3) and contributes to tumor propagation and drug resistance^[71]. To better contextualize clinical impact, KDMs will be further expanded upon in the analysis of therapeutic approaches.

Functionally, PRMTs serve to add methyl groups onto the arginine residues of certain target proteins, thereby disrupting their protein-protein interactions and corresponding downstream cellular processes^[16]. Dichotomized, Type I PRMTs catalyze the mono- and asymmetric di-methylation of arginine, while Type II PRMTs catalyze mono- and symmetric di-methylation of arginine^[41]. In GBM patients, PRMT5 and PRMT1 have historically been overexpressed and negatively associated with overall survival^[72-75]. For example, GBM cells utilize PRMT5 to avoid mTOR inhibition, with *in vivo* inhibition of PRMT5 generally resulting in increased survival in animal models^[72,74,76]. Furthermore, depletion of either PRMT5^[74,76,77] or PRMT1^[75] in intracranial orthotopic mouse xenograft models has demonstrated significant inhibition of tumor growth^[16]. PRMT3 has been linked to metabolic pathway regulation in GBM, specifically preventing ubiquitination of HIF-1 to promote glycolysis. Additionally, PRMT3 knockdown in GSCs has been demonstrated to induce cell cycle arrest and apoptosis, with its inhibition causing decreased tumor growth in xenograft mouse flank models^[78].

Histone acetylation and deacetylation

Akin to their methylating counterparts, lysine acetyltransferases/histone acetyltransferases (KATs/HATs) catalyze the addition of acetyl groups to histone N-terminal lysine residues^[40]. As mentioned previously, acetylation is believed to neutralize the charge of histone tails, thereby weakening histone-DNA and/or internucleosomal interactions^[79]. The result is an increasingly destabilized nucleosome and chromatin structure, which allows nuclear factors such as RNA polymerase II to gain access to the DNA^[40,80,81]. Comparatively, little work has been done in understanding the role of KATs/HATs in GBM; however, it has been identified that KAT6A is upregulated in the course of disease-promoting tumorigenesis through PIK3CA expression and the activation of the PI3K/AKT pathway^[40,82,83]. Counteracting KATs/HATs are the histone deacetylases (HDACs), which are further subdivided into four classes based on yeast ortholog similarities. In GBM specifically, profiling experiments have highlighted significant increases in HDAC1, HDAC6, HDAC7, and HDAC10 expression and similar decreases in HDAC5 and HDAC11 expression as compared to normal brain tissue^[40,84]. Research on individual HDACs has elucidated their contributory roles in GBM. Knockdown of HDAC1 in U87MG xenograft models correlated with a decrease in active extracellular signal-regulated kinase (ERK) and AKT, suggesting an interdependence between HDAC1 activity and the mitogen-activated protein kinase kinase (MEK)/ERK and PI3K/AKT pathways in GBM^[85]. Moreover, HDACs have been associated with chemoradiation and TMZ resistance in GBM through their inhibition of DNA double-strand break repair^[46,86,87]. Particularly, HDAC3 and HDAC1 overexpression in GBM is strongly associated with decreased overall survival in human patients, and its *in vitro* inhibition with RGFP109, a selective HDAC1 and HDAC3 co-inhibitor, led to greater TMZ potency in typically TMZ-resistant A172, U118, U251, and U87 cell lines^[88].

Additionally, acetylated lysines serve as binding sites for bromodomain-containing proteins (such as BRD4), which play crucial roles in regulating gene expression. For instance, BRD4 has been shown to enrich regions of the genome characterized by high acetylation levels, and is implicated in maintaining stem-like properties in glioblastoma cells^[89]. Additionally, BRD4 regulates the self-renewal and tumorigenic potential of glioma-initiating cells by directly interacting with the promoter region of key genes like Notch1, further demonstrating its importance in glioma biology^[90]. This interaction highlights how acetylation not only influences chromatin accessibility but also enables the recruitment of specific transcriptional regulators, contributing to tumor progression and resistance to therapy.

Chromatin remodeling

Chromatin can be modified through various mechanisms involving histone modifiers, histone chaperones, and adenosine triphosphate (ATP)-dependent chromatin remodelers [Figure 1D]. These modifications alter chromatin conformation, which can either enhance or reduce its accessibility to transcription factors and the DNA repair and replication machinery. ATP-dependent chromatin remodelers, which include the switch/sucrose nonfermenting (SWI/SNF), Imitation Switch (ISWI), nucleosome remodeling and deacetylase (NuRD)/Mi-2/Chromodomain Helicase DNA-binding (CHD), INO80, and SWR1 complexes, are particularly influential in this process^[91-94]. They orchestrate the repositioning of nucleosomes, the exchange of histone variants, and play a significant role in GBM drug resistance.

Research has shown that approximately 20% of cancers exhibit alterations in the SWI/SNF gene subunits. The SWI/SNF complex typically functions to slide and remove nucleosomes, thereby regulating chromatin structure to control gene transcription and facilitate DNA replication^[95]. In GBM, GSCs are believed to drive sustained tumor growth, treatment resistance, and recurrence. A recent study by Di Giuseppe *et al.* investigated extracellular vesicles, particularly exosomes and microvesicles, secreted by GSCs. Their findings revealed that stimulation of the ionotropic receptor P2X7 in human GSCs led to significant proteomic changes in the released extracellular vesicles. Specifically, P2X7R activation was associated with increased glioma progression, cell aggressiveness, and migration, and it promoted the secretion of proteins that enhance therapeutic resistance, which include the chromatin remodeling protein RuvB-like 2^[96].

It is thought that chromatin remodelers, especially the SWI/SNF complex, are crucial in maintaining these GSC populations. Evidence suggests that the catalytic bromodomain of SWI/SNF^[97], BRG, is key to maintaining GSCs, with its inhibition sensitizing GSCs to TMZ and carmustine^[98]. Another study by Ji *et al.* identified that the SWI/SNF^[99] subunit Actin-like 6A (ACTL6A) is highly expressed in stem and progenitor cells and supports the progenitor state. ACTL6A promotes the proliferation, invasiveness, and migration of glioma cells by regulating the YAP/TAZ pathway^[99]. Furthermore, modifications to PFI-3, an inhibitor of the BRG1 and BRM catalytic subunits of SWI/SNF, have been shown to increase sensitization to TMZ and bleomycin^[100]. These findings underscore the importance of understanding the chromatin remodeling mechanisms in relation to treatment resistance in GBM.

Non-coding RNA

Non-coding RNAs (ncRNAs) are RNA molecules that are not directly translated into proteins. Still, ncRNAs are a major element of epigenetic mechanisms because of their ability to regulate gene expression. Abnormal ncRNA activity has long been associated with the regulation of tumor onset and progression for various cancers, including GBM, by acting as tumor-suppressing genes or oncogenes^[101]. Importantly, ncRNAs have also been shown to play a critical role in GBM's tendency to become resistant to therapeutic agents, such as TMZ chemotherapy. Several ncRNAs relevant to GBM have been identified through previous research, the dysregulation of which has already been shown to be significantly different both between GBM patients and healthy controls as well as between individuals before and after undergoing tumor resection^[102,103]. These findings show that ncRNAs show promise both as biomarkers and as potential therapeutic targets.

There are strong associations between certain ncRNAs and different malignant characteristics of GBM, though GBM's inherent heterogeneity means that there is some variability in whether particular ncRNAs are over- or underexpressed^[102,103]. The ultimate impact of this dysregulation depends on the tumor microenvironment as a whole. In this context, ncRNAs have been shown to regulate angiogenesis, cell growth, cell cycle progression, apoptosis, tumor invasiveness, and immune evasion, as well as causing treatment resistance^[104].

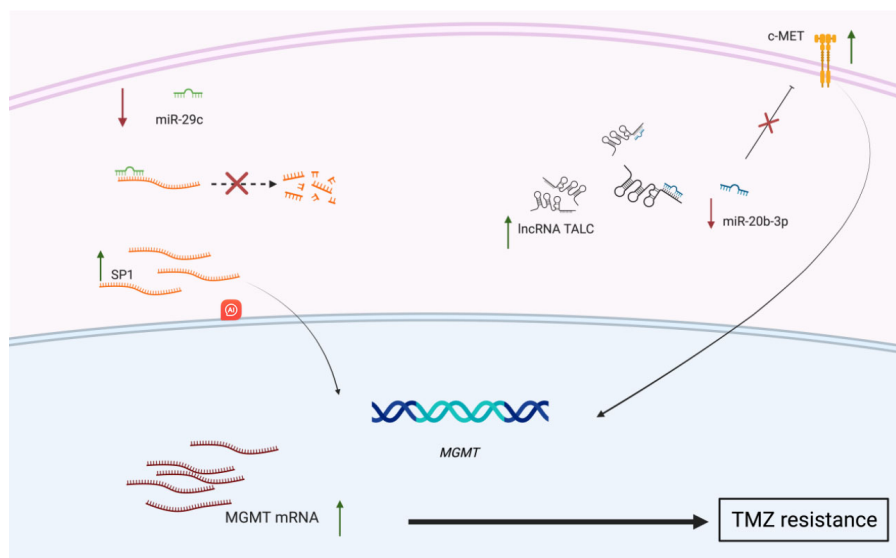


Figure 2. Non-coding RNAs involved in the development of temozolomide resistance in GBM. Under regular conditions, miR-29c binds to the mRNA of SP1 and induces its degradation. However, the downregulation of miR-29c in GBM leads to increased abundance in SP1 mRNA and elevated SP1 protein expression, which in turn induces the expression of the *MGMT* gene. Additionally, the overexpression of the lncRNA TALC leads to a decreased abundance of miR-20b-3p. This attenuates the inhibition that miR-20b-3p exerts over the expression of c-MET, resulting in c-MET overexpression and subsequent downstream signaling to augment *MGMT* expression. The increased *MGMT* expression induced by these mechanisms ultimately augments the GBM cells' ability to resist treatment with TMZ. c-MET: Cellular mesenchymal-epithelial transition factor; GBM: glioblastoma; lncRNA: long non-coding RNA; mRNA: messenger RNA; *MGMT*: O6-methylguanine-DNA methyltransferase; miRNA: microRNA; SP1: specificity protein 1; TMZ, temozolomide.

Regulatory ncRNAs are separated by size. Major categories include micro-RNA (miRNA, around 20 nucleotides), short interfering RNA (siRNA, between 20 and 25 nucleotides), long non-coding RNA (lncRNA, over 200 nucleotides), and circular RNA (circRNA, over 200 nucleotides)^[105]. The defining difference between miRNA and siRNA is that a particular miRNA may have multiple epigenetic targets while being highly specific to one RNA sequence, while siRNAs are only partially complementary to their targets and are preferred for drug discovery^[106,107]. In both cases, the ncRNA causes gene silencing by binding to a complementary segment of an mRNA molecule, prohibiting translation. lncRNAs modulate gene transcription and the stability of mRNAs, having the potential to serve several roles as scaffolds, decoys, or transcription signal transmitters^[108]. circRNA and lncRNAs can act as “sponges” for miRNA, binding to them and thus inhibiting the action of miRNA. circRNA molecules are similar to lncRNAs in length but take on a circular structure because their 3' end is covalently bonded to the 5' end^[109].

micro-RNA in glioblastoma treatment resistance

Abnormal regulation of miRNAs associated with GBM leads to cascading epigenetic effects, contributing to treatment resistance (summarized in Table 1). For instance, miR-152-3p typically targets DNMT1 and the methylation of NF2, making it important for glioma apoptosis^[110]. In GBM, miR-152-3p is downregulated, lessening glioma apoptosis. Similarly, miR-29c indirectly targets *MGMT* that supports a positive response to TMZ, and is downregulated in GBM, leading to TMZ resistance^[111] [Figure 2]. MiR-129-5p also targets DNMT3a and is downregulated in GBM, causing TMZ resistance and providing a good prognosis marker^[112]. In contrast, MiR-10b-5p targets a TET2 pathway that induces tumor progression and stemness features and is upregulated in GBM^[113].

MiR-101-3p is downregulated in GBM, but this miRNA and its targets (EZH2 and H3K27me3) have already been associated with a therapeutic strategy that targets proliferation, migration, and angiogenesis^[114-116]. MiR-22 targets SIRT1 and its downregulation allows for tumor cell proliferation, motility, and invasion^[117].

Table 1. Summary of the non-coding RNAs, along with their molecular targets, that are involved in the treatment resistance of glioblastoma

RNA	Expression pattern in GBM	Target	Result	Reference
Micro RNA				
miR-152-3p	Downregulated	DNMT1	Reduced apoptosis	[110]
miR-29c	Downregulated	MGMT	TMZ resistance	[111]
miR-129-5p	Downregulated	DNMT3a	TMZ resistance	[112]
miR-10b-5p	Upregulated	TET2	Tumor progression and stemness	[113]
miR-101-3p	Downregulated	EZH2	Proliferation, migration, and angiogenesis	[115]
miR-22	Downregulated	SIRT1	Proliferation, motility, and invasion	[117]
miR-9	Upregulated	PTCH1	TMZ resistance	[118]
miR-223	Upregulated	PAX6	TMZ resistance	[114,116]
miR-155-3p	Upregulated	Six1	TMZ resistance	[119]
miR-93/193	Upregulated	Cyclin D1	TMZ resistance	[120]
Long non-coding RNA				
AC016405.3	Downregulated	miR-19a-5p//TET2	Tumor progression	[121]
HOTAIR	Upregulated	PRC2 and EZH2	Cell cycle progression	[122]
LINC00461	Upregulated	miR-485-3p	Cell cycle progression	[123]
TALC	Upregulated	miR-20b-3p//MGMT	TMZ resistance	[124]
SNHG12	Upregulated	miR-129-5p//MAP-ERK	TMZ resistance	[125]

DNMT: DNA methyltransferases; MGMT: O⁶-methylguanine-DNA methyltransferase; TET: ten-eleven translocation; EZH: enhancer of zeste homolog; SIRT: silent information regulator sirtuin 1; PTCH1: patched 1; Six1: sine oculis homeobox 1; PAX6: paired box 6; MAP-ERK: mitogen-activated protein kinases- extracellular signal-regulated kinases; PRC: polycomb repressive complex.

Moreover, one major form of treatment resistance in GBM is resistance to TMZ. The most well-known predictor of TMZ resistance is *MGMT*. However, many miRNAs have been found to be directly involved in resistance to TMZ treatment, including miR-9 (targeting PTCH1)^[118], miR-223 (targeting PAX6)^[114,116], miR-155-3p (targeting Six1)^[119], and miR-93/193 (targeting Cyclin D1)^[120].

lncRNA in GBM treatment resistance

Similarly, lncRNA alterations have been widely implicated in the progression and treatment resistance of GBM (summarized in Table 1). For example, the downregulation of AC016405.3 undermines tumor suppression by disrupting DNA methylation and TET enzymes^[121]. In this case, AC016405.3 works via miR-19a-5p to regulate TET2 and suppress tumors^[121]. Conversely, HOTAIR is upregulated in GBM. HOTAIR targets chromatin-modifying complexes like PRC2 and regulates cell cycle progression through EZH2. Moreover, HOTAIR-targeting histone demethylase and LSD1 are upregulated cell cycle regulators that work through EZH2 to induce apoptosis^[122]. LINC00461, another upregulated lncRNA, plays a role in GBM progression by targeting miR-485-3p which is critical to cell cycle regulation^[123].

There are several lncRNAs relevant to treatment resistance, such as lncRNA-TALC and SOX2OT, both of which promote TMZ resistance when upregulated. lncRNA-TALC does this by promoting *MGMT* expression and, thus, TMZ resistance^[124] [Figure 2]. Moreover, the upregulation of SNHG12 causes TMZ resistance through the targeting of DNA methylation of *MGMT* and even serves as a prognostic marker^[125].

EPIGENETIC-BASED HETEROGENEITY AND PLASTICITY

GBM tumors are known to be among the most heterogeneous tumors classified by the 2021 World Health Organization (WHO) classification of central nervous system (CNS) tumors^[126,127]. Heterogeneity refers to the existence of distinct subpopulations of cells within a tumor, each exhibiting diverse genotypes and

phenotypes^[126,128]. This heterogeneity seen in GBM largely contributes to its resistance to treatment. This heterogeneity is driven by the presence of a variety of epigenetic profiles that exist on the intratumoral and intertumoral levels.

When triggered by epigenetic modifications and the tumor microenvironment (TME), normal neuronal stem cells, which are normally destined to become oligodendrocytes, ependymal cells, or astrocytes, can develop into cancer stem cells (CSCs) that are referred to as GSCs^[126,129]. One of the main features that characterizes these GSCs is their plasticity, which is defined as “morphological and functional flexibility”^[129]. This flexibility allows GSCs to adapt to different microenvironments and persist even under harsh conditions and in the presence of therapeutic agents. In this context, plasticity also refers to a cell’s ability to interconvert from one cellular sub-state to another. This plasticity is mainly regulated by epigenetic modulations occurring in the genome, altering the expression of regulatory genes in GSCs, and driving the interchange between states in response to stimuli from the TME^[126,127]. Herein, according to the plasticity model formulated to explain the heterogeneity within GBM and built upon the CSC model: “CSCs are the source of tumor initiation and heterogeneity...CSCs can interconvert between stem cell and differentiated states”^[130].

Specifically, intratumoral heterogeneity (inside of a single tumor) is maintained by epigenetic modulations, which include the previously discussed methylation of the *MGMT* gene and decreased chromatin accessibility^[130]. Additionally, intratumoral heterogeneity is seen within GBM at the level of cellular subtypes^[131]. Specifically, of the four subtypes identified within GSCs, the proneural (PN-GSC) and mesenchymal (Mes-GSC) subtypes are the most studied^[131]. The PN-GSC subtype is detected in secondary GBMs and young patients, while the mesenchymal (Mes-GSC) subtype is found more often in older patients and reveals a primary and secondary GBM origin^[131]. Mes-GSCs are known to be more aggressive phenotypes which contribute to worse prognoses in comparison to PN-GSCs. Mes-GSCs exhibit a more proliferative phenotype due to their expression of CD44, YKL40, Lyn, WT1, and BCL2A1, which are associated with angiogenesis and enhanced cell survival by counteracting apoptosis, inflammation, and cell migration/invasion^[129,130]. In contrast, PN-GSCs express CD133, EXH2, Olig2, Sox2, and Notch1. These genes are involved in homeostasis, the cell cycle, DNA repair, and the activation of Notch and PDGF receptor signaling pathways, which impart a better prognosis^[132].

Compounded with genetic and epigenetic changes, the TME induces the dynamic transitions between GSCs^[127]. In fact, heterogeneity also exists at the level of the TME and includes the hypoxic niche, the perivascular niche, and the invasive niche^[133,134]. Herein, hypoxia is defined as a state in which oxygen is insufficient to maintain homeostasis^[135]. Therefore, the hypoxic niche is characterized by low oxygen levels in brain tissues and the overexpression of HIFs. These factors increase the growth of HSCs and promote angiogenesis and chemoresistance^[130]. Hypoxia offers phenotypic variability and functional characteristics to GSC subpopulations to promote adaptability to these hypoxic regions^[133]. The invasive microenvironment, called the perivascular niche, is characterized by its collaborative network of cancer cells, endothelial cells, pericytes, astrocytes, and tumor-associated macrophages that regulate cell migration/invasion into surrounding healthy tissue^[134].

Hence, GBM plasticity is attained through epigenetic modifications such as histone modification, DNA methylation, and chromatin remodeling in GBM cells^[127]. The plasticity of GBM is characterized by transitions between the proneural (less active) and mesenchymal (more active) subtypes, as well as the ability to adapt to various microenvironments. For instance, after treatment with TMZ, GSCs have been observed to adapt toward drug-resistant states, forming populations of heterogeneous drug-resistant cells. Due to the epigenetic plasticity of these cells, they are more likely to continue alternating between stem cell progenitor

Table 2. Summary of the clinical trials using epigenetic modifiers, either in isolation or in combination. Based on data from clinicaltrials.gov

Agent	Trials	Type/Design	Intervention	Status
Valproic Acid	NCT00302159	Phase II	VPA +/- TMZ	Completed, published ^[137]
Abexinostat (PCI-24781)	NCT05698524	Phase I	Abexinostat +/- TMZ	Recruiting
	NCT01266031	Phase I/II	Vorinostat +/- bevacizumab	Completed, results posted on clinicaltrials.gov
	NCT02420613	Phase I	Vorinostat and temsirolimus +/- radiation therapy	Active, not recruiting
	NCT00555399	Phase I/II	Vorinostat, isotretinoin and temozolomide	Terminated, no sponsor funding for continuation of trial
	NCT01189266	Phase I/II	Vorinostat and radiation therapy	Completed, results posted on clinicaltrials.gov
	NCT00268385	Phase I	Vorinostat and temozolomide	Active, not recruiting
	NCT01738646	Phase II	Vorinostat +/- bevacizumab	Completed, results posted on clinicaltrials.gov
	NCT01378481	Phase I	Vorinostat and fractionated stereotactic body radiation therapy	Terminated
	NCT00939991	Phase I/II	Vorinostat, bevacizumab and temozolomide	Completed, results posted on clinicaltrials.gov
Vorinostat	NCT01236560	Phase II/III	Vorinostat, temozolomide, or bevacizumab in combination with radiation therapy followed by bevacizumab and temozolomide	Completed, results posted on clinicaltrials.gov
	NCT01110876	Phase I/II	Vorinostat, erlotinib and temozolomide	Terminated, unanticipated toxicities
	NCT00641706	Phase II	Vorinostat and bortezomib	Completed, results posted on clinicaltrials.gov
	NCT00731731	Phase I/II	Vorinostat, temozolomide, and radiation therapy	Completed, results posted on clinicaltrials.gov
	NCT00238303	Phase II	Vorinostat	Completed, results posted on clinicaltrials.gov
	NCT03426891	Phase I	Pembrolizumab and vorinostat	Completed, no results posted
	NCT00762255	Phase I	Vorinostat, bevacizumab & irinotecan	Completed, results posted ^[136]
	NCT00994500	Phase I	Vorinostat and bortezomib	Completed, no results posted
	NCT00217412	Phase I	Vorinostat +/- isotretinoin	Completed, no results posted
	NCT01076530	Phase I	Vorinostat and temozolomide	Completed, no results posted

VPA: Valproic acid; TMZ: temozolomide.

states and more differentiated states, depending on the prevailing conditions and microenvironment.

THERAPEUTIC APPROACHES WITH EPIGENETIC TARGETS

This section explores the potential targeting strategies that can help attenuate the therapeutic resistance of GBM. Table 2 summarizes epigenetic modifiers that have progressed to clinical trials and describes the progress/results of these trials. ^[136,137]

Targeting DNA methylation

Therapeutic approaches targeting DNA methylation represent an encouraging avenue for the treatment of GBM. By reversing aberrant DNA methylation patterns associated with tumor suppressor gene silencing, these strategies aim to inhibit tumor growth, overcome therapeutic resistance, and improve outcomes for GBM patients.

DNMT inhibitors, such as azacytidine and decitabine, have been studied for their potential in treating GBM^[138,139]. These compounds can counteract abnormal DNA hypermethylation commonly seen in GBM, resulting in the reactivation of tumor suppressor genes silenced by excessive promoter methylation^[140]. Clinical trials are currently investigating the effectiveness of DNMT inhibitors, either alone or combined with other therapies, particularly TMZ, in managing GBM^[141].

Moreover, innovative genome editing techniques like clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 hold great potential for precisely modifying DNA methylation patterns in GBM. The CRISPR/Cas9 system is a type II CRISPR system that consists of three key constituents: transactivating crRNA (tracrRNA), an endonuclease (Cas9), and CRISPR RNA (crRNA). When crRNA pairs with tracrRNA, they create a molecule known as single-guide RNA (sgRNA). This sgRNA guides Cas9 to bind to the target sequence and cleave foreign DNA^[142]. Han *et al.* used the CRISPR /Cas9 system to knock out the *MGMT* gene, thus reducing resistance to TMZ^[143]. Similarly, to achieve the same effect of reduced TMZ resistance, Tong *et al.* used CRISPR /Cas9 to knock out the *MUC1* gene^[144]. This targeted modification could potentially reverse tumor suppressor gene silencing at specific genomic sites, paving the way for more tailored therapies for GBM patients^[142]. Nevertheless, further investigation is necessary to refine the delivery methods and enhance the specificity of DNA methylation editing tools for their clinical implementation in GBM.

Yao *et al.* introduced a groundbreaking approach termed Methylated Oligonucleotide-Directed DNA Methylation, demonstrating its efficacy in hepatocellular carcinomas. They synthesized a methylated oligonucleotide (MON) complementary to the *IGF2* promoter, inducing hypermethylation and suppressing *IGF2* mRNA expression. This technique involves phosphonothioate modification to replace cytosine residues with methyl groups, resulting in 5mC formation. The MON comprises an inactivating element (IE) and a guiding element (GE). The GE directs the IE to specific loci, where a modified hemimethylated CpG hairpin structure recruits DNMT1, initiating methylation. This process mimics a replication fork, facilitating continuous DNMT1-mediated methylation. Upon dissociation, the methylated strand pairs with the unmethylated strand, creating a second hemimethylated substrate for DNMT1 to methylate, ultimately inducing site-specific gene methylation^[145]. In a similar fashion, small non-coding RNAs can also be used to direct methylation where desired. They can guide DNA methylation by forming RNA-DNA hybrids with complementary genomic sequences through microRNA (miRNA), small interfering RNA (siRNA), or piwi-interacting RNA (piRNA) mediated pathways resulting in recruitment of DNMTs^[146].

Hence, identifying predictive biomarkers linked to DNA methylation patterns in GBM can help tailor treatment strategies to individual patients. Currently, the *MGMT* methylation status serves as a biomarker preceding standard chemoradiation. It is hypothesized that *MGMT* methylation status could also inform immunotherapy approaches. Evidence suggests that patients with *MGMT*-methylated tumors exhibit significantly enhanced survival rates compared to those with unmethylated *MGMT* in GBM vaccine therapy trials. Additionally, other potential biomarkers such as *PTEN* and *IDH* have been explored^[147-149]. Utilizing biomarker-guided approaches enables the selection of patients who are likely to derive benefits from epigenetic therapies, thereby enhancing treatment outcomes while minimizing adverse effects. The integration of biomarker information into the clinical decision-making processes has the potential to refine the precision and efficacy of epigenetic therapies for GBM.

Another promising avenue involves targeting bromodomain and extraterminal (BET) proteins, which play a key role in regulating gene expression through their interaction with acetylated histones. Specifically, inhibition of BET proteins, such as BRD4, has been shown to sensitize glioblastoma cells to TMZ by downregulating the expression of *MGMT*, a DNA repair enzyme responsible for repairing TMZ-induced

DNA damage. *MGMT* is often overexpressed in GBM and its high levels contribute to the resistance of tumor cells to TMZ therapy^[150].

Targeting histone modification

Histone methylation and demethylation

Research into the therapeutic possibilities of histone lysine demethylase (KDM) has primarily developed over the last 15 years. In 2011, Singh *et al.*^[151] established the *in vitro* efficacy of KDM1 inhibition in sensitizing GBM to HDAC inhibitors and subsequent interest has led to an increase in experimental and clinical trials of KDM1 inhibitors. Inhibiting KDM1A (LSD1) with either NCL-1 or NCD-38 has been shown to decrease neurosphere formation and cell viability in GSCs, while also promoting differentiation, increasing endoplasmic reticulum stress, inducing apoptosis, and enhancing the efficacy of TMZ^[152,153]. Additionally, another KDM1 inhibitor, tranylcypromine (TCP), combined with vorinostat, has resulted in increased apoptosis in the U87 glioma cell line *in vivo*^[151]. Clinically, many irreversible LSD1 inhibitors have undergone cancer therapy assessment, including TCP, Iadademstat (ORY-1001), Vafidemstat (ORY-2001), GSK-2879552, Bomedemstat (IMG-7289), and INCB059872^[154]. Due to the enduring effects of irreversible inhibitors, many reversible LSD1 inhibitors have also been researched and documented, although only two of them (Seclidemstat (SP-2577) and Pulrodemstat (CC-90011)) have progressed to clinical trials. To date, only BEA-17, a degrader of LSD1 and its cofactor, coREST, has been granted the orphan drug designation by the United States Food and Drug Administration (FDA) for use in GBM.

Due to the previously described effects of KDM2A, it is theorized that its inhibition could augment the immunotherapeutic response against GBM, although further research is required^[46]. This is primarily due to a lack of mechanistic understanding of KDM2A function and uncertainty regarding the *in vivo* efficacy of its inhibition. In contrast, the prevalence of the KDM4 lysine demethylase family in clinical GBM has allowed efforts to yield great strides in mechanistic understanding. In 2018, Voon *et al.* described the inhibition of KDM4 caused by pediatric GBM H3.3 G34R mutants, and the resultant epigenetic dysregulation^[155]. Subsequent studies conducted in 2021 by Lee *et al.*^[156] revealed the particular significance of KDM4C in GBM tumorigenesis and p53 and c-MYC regulation. Specifically, KDM4C knockdown in U87 and U251 GBM cell lines has led to reduced colony formation, decreased c-MYC expression, and increased p53 levels. Furthermore, the inactivation of KDM5A through JIB 04, a pan-KDM inhibitor, or CP1445, a KDM5A-selective inhibitor, was found to efficiently restore TMZ sensitivity in adaptively resistant GBM cells^[46,157,158]. When supplemented with the KDM6B inhibitor GSK-J4, JIB 04 was found to further increase potency against TMZ-resistant GBM cells^[46,158].

Histone acetylation and deacetylation

Most clinical attempts at GBM treatment through epigenetic modification have been directed toward the interplay between acetylation and deacetylation, particularly with regard to HDAC inhibitors and their ability to sensitize GBM to chemotherapeutic and radiotherapeutic treatments. Notably, almost all patients with GBM are susceptible to recurrence—a quality attributed to poor blood-brain barrier (BBB) drug permeability, intratumor heterogeneity, intrinsic GBM treatment resistance, and non-specific agent toxicities^[159,160]. However, there are several HDAC inhibitors capable of penetrating the BBB and serving in an anti-GBM role through the upregulation of p21Waf1/Cip1, a cell-cycle inhibitor^[159]. For example, *in vitro* treatment with the pan-HDAC inhibitor, phenylbutyrate, suppressed the proliferation of the LN-229 GBM cell line^[161]. Accordingly, the application of romidepsin produced synergistic results in the U251MG cell line by reducing the respective anti-apoptotic protein Bcl-2^[159,162]. Additionally, HDAC inhibitors have proven capable of regulating GBM angiogenesis through inhibition of growth factors such as VEGF and EGFR or impeding upon vascular mimicry^[159].

BET bromodomain inhibition (using HMBA) combined with MEK inhibition as a potential therapeutic strategy for GBM is also being explored. By targeting the BET proteins, which regulate key genes involved in tumor growth, and inhibiting the MEK-ERK signaling pathway, HMBA (BET bromodomain inhibitor) demonstrated enhanced antitumor effects compared to either approach alone. The synergistic combination led to reduced tumor cell proliferation, increased cell death, and suppression of survival pathways in preclinical models, suggesting that this dual inhibition could overcome some of the limitations of current therapies^[163].

Preclinically, vorinostat, suberoylanilide hydroxamic acid (SAHA), Trichostatin A (TSA), and valproic acid (VPA) have significantly precipitated GSC autophagy, reduced proliferation, and stimulated differentiation^[159,164,165]. *In vitro* studies conducted by Urdiciain *et al.* solidified the abilities of HDAC6-selective inhibitors such as ACY-1215, tubastatin A, and CAY10603 to overturn TMZ resistance in patient-derived T98G and LN405 GBM cell lines^[166]. In clinical trials, there have been several studies analyzing the synergistic effects of HDAC inhibitors such as vorinostat, panobinostat, and VPA with TMZ, bevacizumab, and radiation; however, none have progressed past trial phase II^[159]. Currently ongoing are studies attempting to analyze the efficacy of various HDAC inhibitors, such as Abexinostat (PCI-24781), in the treatment of recurrent GBM. While promising, the extensive use of HDAC inhibitors within the clinical setting is limited due to a generally poor understanding of the relationship between the toxicity and pharmacokinetic properties of these inhibitors. Furthermore, identification of patients likely to respond to HDAC inhibitor treatment is difficult due to GBM heterogeneity and epigenetic profiling, compounded by the challenges of converting the promising preclinical experiments into potential therapeutic regimens for clinical trials^[159].

Targeting chromatin remodeling

The dysregulation of chromatin remodeling complexes contributes to the aberrant gene expression patterns and cellular behaviors that are observed in GBM. In a recent study by Sun *et al.*, a GBM-specific epigenetic mechanism was discovered where the chromatin regulator bromodomain-contain protein 8 (BRD8) helped to maintain histone variant H2AZ at p53 targets, thereby enhancing chromatin accessibility and generating repressive chromatin state, preventing the tumor suppressor activity of p53. They found that targeting the BRD8 displaces H2AZ, allowing for the activation of p53 and leading to subsequent tumor suppression and cell cycle arrest in the TP53WT GBM cell line^[167]. These findings suggest that targeting BRD8-mediated chromatin remodeling in GBM presents a promising therapeutic strategy for overcoming the epigenetic barriers of p53 activation and potentially improving patient outcomes.

Further studies have also implicated the role of poly (ADP-ribose) polymerase (PARP) inhibitors as a potential therapeutic target in GBM. Previously, PARP1 has been implicated in the stabilization of DNA replication forks, single-stranded and double-stranded DNA breaks, as well as the modulation of chromatin structure^[168,169]. Since PARP1 is a target of DNA damage machinery, it has been an attractive target to help generate synthetic lethality and potentiate the effects of chemotherapy and radiotherapy^[170,171]. PARP inhibitors (PARPi), in particular Olaparib, Niraparib, and Rucaparib, have already gained approval from the FDA and have been shown to improve survival rates in patients with ovarian cancer, breast cancer, and prostate cancer, especially those with BRCA deficiencies^[172-174]. PARPi have also been shown to play an important role in the modulation of chromatin structures that facilitate DNA repair as they will act to PARylate histone tails, resulting in a relaxed chromatin state and the removal of nucleosomes from the DNA^[168,175]. As such, the usage of PARPi has recently garnered interest in the treatment of GBM. In the OPARTIC trial, a phase I dose escalation study of Olaparib in combination with TMZ in patients with relapsed GBM, the pharmacokinetics of Olaparib were investigated. The results showed that of the patients who received the drug, penetration was detected in 71/71 of the tumor core specimens, demonstrating its

consistent ability to cross the BBB^[176]. Therefore, the use of PARPi presents an exciting therapeutic approach with numerous other clinical trials currently evaluating their effects in the treatment of GBM.

Targeting non-coding RNAs

ncRNAs as biomarkers

Some ncRNAs could be used as biomarkers to predict the progression or presence of the disease; these include miRNA-21, miRNA-26a, miR-128, and miR-342, among others. These prognosis markers could be extremely helpful in the detection and diagnosis of GBM. Early detection could reduce patient mortality and prolong survival. Additionally, other ncRNAs predict the likelihood of GBM resistance to certain treatments. In the future, this could be helpful in the clinical setting when devising treatment plans. Studies by ParvizHamidi *et al.* on miRNA-21 and miRNA-26a and by Wang *et al.* on miR-128 and miR-342 identified those miRNAs as important biomarkers^[102,103]. Specifically, expression was quantified in people without GBM and in GBM patients before and after surgery. Before surgery, there was a large difference in the expression of these RNAs between patients with GBM and healthy subjects; however, this difference between groups was ameliorated after surgical resection. This implies that the tumor was driving the overexpression of these miRNAs, and their presence returned to normal once the tumor burden was reduced. For patients with GBM, high expression of miR-1258, miR-935, and miR-128-3p was associated with better overall survival, and the same association was found for low expression of miR-542-3p and miR-221/222^[102,103]. These patterns could have great clinical benefits-surveilling ncRNA expression in a patient could provide insights into their prognosis and possible risk of treatment resistance. This could help inform treatment plans to maximize treatment efficacy based on the individual patient's expression patterns.

ncRNA therapies and delivery systems

The sheer number of ncRNAs means that there are many possible epigenetic targets to be explored in the therapeutic context^[177]. Herein, miR-124-2, miR-135a-2, and let-7i were found to be the most useful miRNAs with clinical relevance in a study screening around 600 different miRNAs. Alternatively, miR-17-3p, miR-340, and miR-222 are critical miRNAs that modulate GBM cell viability *in vitro* and *in vivo*^[177]. These two lists of relevant miRNAs do not cover the full scope of possibilities for targets of therapeutic treatment. Some other targets include several miRNAs that have been found to promote GBM sensitivity to TMZ, such as miR-198 (*MGMT*), miR-101 (*GSK-3beta*), miR-1268a (*ABCC1*), miR-381 (*ABCG2*, *ABCC3*, *ABCC5*), miR-137 (*LRP6*), miR-126-3p (*SOX2*), and miR-128-3p (*c-Met*, *EMT*)^[178].

Other therapies utilize small-interfering RNA (siRNA) to target genes (such as VEGF, EGFR, STAT3, and *ETDL1*) that promote GBM progression and downregulate them, suppressing their oncogenic effect^[104,106]. Other siRNAs include Livin-siRNA, which enhances TMZ sensitivity through the inhibition of MRP1, and Hsp27-siRNA, which induces apoptosis. To improve delivery efficacy, nanoparticles (NPs) are being utilized for delivery formulations. Such nanoparticles include iron oxide nanoparticles, liposomes, and other compounds specifically developed to deliver a particular siRNA^[179].

ncRNAs work cooperatively, and this property can be leveraged through specific combinations to enhance their overall effect. For example, the miR-Combo formulated by Bassot *et al.* used a combination of miR-17-3p, miR-222, and miR-340, which significantly decreased cell viability compared to non-targeting scrambled miRNA^[180].

Delivery systems

The overarching challenge in designing these therapies lies in delivering these treatments to the targets in the brain. Some obstacles to delivery include the breakdown of therapeutic agents in the blood, poor molecular stability, clearance by the kidneys, and the inability to penetrate relevant membranes. The most striking challenge in the context of GBM is the existence of the BBB^[181]. Even if a drug can get through the BBB, it

must also pass through the cell membrane to elicit any effect.

Several drug-delivery systems are being studied to improve the efficacy of GBM treatment. Some of these methods include the use of nanoparticles (NPs), which include inorganic NPs, polymeric NPs (PNPs), and lipid-based NPs (LNPs)^[104]. NP delivery systems can provide both controlled drug release and tissue specificity while protecting miRNAs from systemic degradation, ensuring the payload remains intact. The use of synthetic RNA can increase bioavailability^[106], and using siRNA rather than miRNA can counteract cytotoxicity due to target specificity. Other possible delivery systems include stem cell-derived exosomes, bacterial toxins, and viral vectors to improve cell membrane penetration and successful delivery^[104].

CONCLUSIONS AND FUTURE DIRECTIONS

The alterations in DNA methylation status, histone methylation and acetylation status, chromatin architecture, and expression of non-coding RNAs all play critical roles in modifying gene expression in GBM and promoting a resistant phenotype. It is imperative to study the intricate interactions between the epigenetic profiles of GBM cells and their ability to adapt to different therapeutics, along with the significant intratumoral and intertumoral heterogeneity that governs these interactions. Achieving a better understanding of these mechanisms and processes can inspire novel avenues to overcome treatment resistance and augment the efficacy of existing and emerging therapeutics. Despite a long history of investigation into epigenetic control and rewiring, further research is still required to safely and effectively navigate the transformation of these mechanisms into translatable therapies and tractable targets that can be integrated into the clinical management of GBM and offer superior outcomes to patients diagnosed with this disease. Nevertheless, it is important to note that tumorigenesis is the result of the combination of many epigenetic events. Additionally, the vast heterogeneity that is present at the intratumoral and intertumoral levels further complicates the issue. Small molecule targeting of one subset of epigenetic regulation may not lead to complete tumor eradication. The development of effective therapeutic modalities will require consideration of this complex interplay between various epigenetic events and signaling pathways that drive GBM progression.

DECLARATIONS

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Conflicts of Interest

Tyler B has research funding from NIH. Therapeutics A Inc. has licensed one of her patents, and she holds stock in Peabody Pharmaceuticals* (*including equity or options). The other authors declared that there are no conflicts of interest.

Availability of data and materials

Not applicable.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Mechanisms underlying reversed TRAIL sensitivity in acquired bortezomib-resistant non-small cell lung cancer cells

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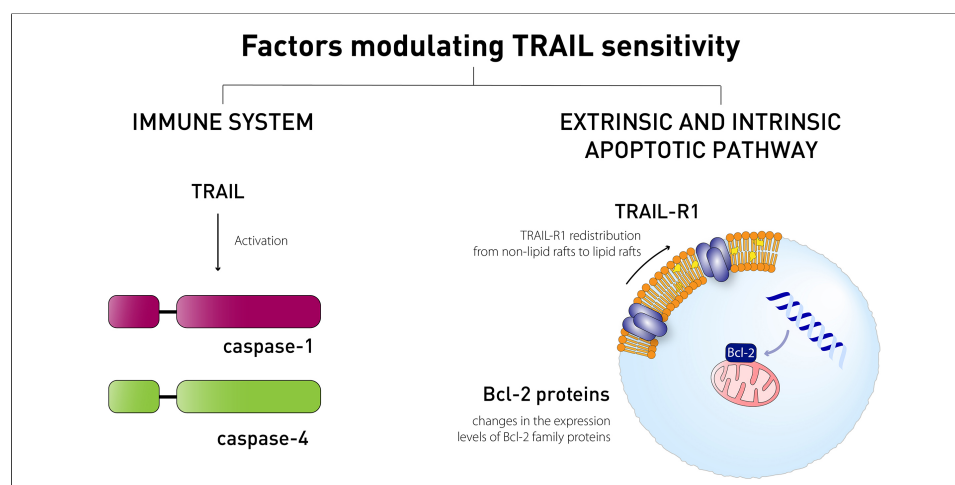
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Abstract

Aim: The therapeutic targeting of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) death receptors in cancer, including non-small cell lung cancer (NSCLC), is a widely studied approach for tumor selective apoptotic cell death therapy. However, apoptosis resistance is often encountered. The main aim of this study was to investigate the apoptotic mechanism underlying TRAIL sensitivity in three bortezomib (BTZ)-resistant NSCLC variants, combining induction of both the intrinsic and extrinsic pathways.

Methods: Sensitivity to TRAIL in BTZ-resistant variants was determined using a tetrazolium (MTT) and a clonogenic assay. A RT-qPCR profiling mRNA array was used to determine apoptosis pathway-specific gene expression. The expression of these proteins

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was determined through ELISA assays and western Blotting, while apoptosis (sub-G1) and cytokine expression were determined using flow cytometry. Apoptotic genes were silenced by specific siRNAs. Lipid rafts were isolated with fractional ultracentrifugation.

Results: A549BTZR (BTZ-resistant) cells were sensitive to TRAIL in contrast to parental A549 cells, which are resistant to TRAIL. TRAIL-sensitive H460 cells remained equally sensitive for TRAIL as H460BTZR. In A549BTZR cells, we identified an increased mRNA expression of *TNFRSF11B* [osteoprotegerin (OPG)] and caspase-1, -4 and -5 mRNAs involved in cytokine activation and immunogenic cell death. Although the OPG, interleukin-6 (IL-6), and interleukin-8 (IL-8) protein levels were markedly enhanced (122-, 103-, and 11-fold, respectively) in the A549BTZR cells, this was not sufficient to trigger TRAIL-induced apoptosis in the parental A549 cells. Regarding the extrinsic apoptotic pathway, the A549BTZR cells showed TRAIL-R1-dependent TRAIL sensitivity. The shift of TRAIL-R1 from non-lipid into lipid rafts enhanced TRAIL-induced apoptosis. In the intrinsic apoptotic pathway, a strong increase in the mRNA and protein levels of the anti-apoptotic myeloid leukemia cell differentiation protein (Mcl-1) and B-cell leukemia/lymphoma 2 (Bcl-2) was found, whereas the B-cell lymphoma-extra large (Bcl-xL) expression was reduced. However, the stable overexpression of Bcl-xL in the A549BTZR cells did not reverse the TRAIL sensitivity in the A549BTZR cells, but silencing of the BH3 Interacting Domain Death Agonist (BID) protein demonstrated the importance of the intrinsic apoptotic pathway, regardless of Bcl-xL.

Conclusion: In summary, increased sensitivity to TRAIL-R1 seems predominantly related to the relocalization into lipid rafts and increased extrinsic and intrinsic apoptotic pathways.

INTRODUCTION

Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancer cases, and it is associated with a very poor 5-year survival rate (approximately 16%)^[1-3]. Although immunotherapy has shown considerable benefits for a subpopulation of NSCLC patients^[4,5], there is still a need for novel targeted agents, including those targeting apoptosis. Apoptosis is required for the depletion of redundant, damaged, or transformed cells, such as viral-infected or malignant cells. However, in most tumor cells, the ability to activate apoptosis is inhibited due to the overexpression of pro-survival proteins and/or loss of proapoptotic proteins^[6,7]. Therefore, the specific activation of apoptosis in tumor cells is a potent strategy to treat cancer. Currently, several tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-based drugs have been or are being investigated for proapoptotic activity in clinical phase II and III trials in NSCLC. One of the major aims of these trials is to test the induction of the immune response against the tumor by TRAIL, although the main problems of TRAIL-based therapeutics are resistance, poor pharmacokinetics, and short half-life^[8,9]. Although apoptosis is an immunologically silent process, there are several links combining it with immune response, e.g., caspase-8^[10,11]. In addition, chemo- and radiotherapy, as well as growth factor receptor inhibitors targeting epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase-echinoderm microtubule-associated protein-like 4 fusion (ALK-EML), and neurotrophic tyrosine receptor kinase (NTRK), are extensively being used for the treatment of NSCLC, and these treatments also induce apoptosis. Thus, the current treatment modalities trigger at least the apoptotic cell death pathway. Therefore, more insight into the resistance mechanism of apoptosis induction will help to improve the current therapy^[10].

The initiation of apoptosis can be regulated via extrinsic and intrinsic apoptotic pathways, which will ultimately result in the activation of the effector caspases^[10]. The activation of the extrinsic apoptotic pathway is achieved by the binding of TNF family members to their associated receptors. Several variants of TRAIL have been or are being investigated for their clinical benefit in phase I, II and III clinical trials (clinicaltrials.gov^[12-15]). TRAIL agonists can bind to the death receptors TRAIL-R1 and TRAIL-R2 and the decoy receptors TRAIL-R3 and TRAIL-R4. The activation of TRAIL-R1 and TRAIL-R2 results in the

trimerization of the receptors and the formation of the intracellular death-inducing signaling complex (DISC) involving Fas-associated death domain (FADD) and caspase-8. This will lead to the activation of caspase-8, which, in turn, can activate effector caspase-3 directly or, alternatively, via the cleavage of BH3 Interacting Domain Death Agonist (BID) into a truncated form, tBID. Truncated BID facilitates the localization of Bak and Bax into the mitochondrial membrane, thereby initiating the intrinsic apoptotic pathway, resulting in the release of cytochrome C and SMAC/DIABLO. Cytochrome C is required for the formation of the apoptosome, where caspase-9 will be activated, leading to the activation of caspase-3, resulting in apoptosis^[16-18]. Apart from the primary DISC, a secondary signaling complex can be formed containing receptor-interacting protein kinase 1 (RIPK1) and TNF receptor-associated factor 2 (TRAF2). This secondary complex can activate several protein kinase pathways that promote cell proliferation, including the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which can enhance the expression of a number of anti-apoptotic proteins, such as cellular FLICE-like inhibitory protein (c-FLIP) and X-linked inhibitor of apoptosis protein (XIAP)^[19,20].

Furthermore, the distribution of the TRAIL receptors within lipid rafts forms an activated DISC and induces cell death, whereas localization in the non-lipid rafts recruits the secondary complex, thereby initiating cell survival^[21-24]. More recently, β -catenin was found to induce the redistribution of the cell surface expression of TRAIL-R1 and -R2, leading to increased caspase-3/8 activation and apoptosis, thus enhancing TRAIL sensitivity^[25]. Other features that contribute to TRAIL resistance consist of mutations in the receptors^[26,27] or O-glycosylation of the intracellular part of the receptor^[28]. In addition, high levels of c-FLIP^[29] and phosphoprotein enriched in diabetes (PED)^[30] have been described to interfere with the formation of DISC, thereby hampering the apoptotic pathway. Further downstream in the apoptotic pathway, resistance can be dependent on high expression levels of the anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2, B-cell lymphoma-extra large (Bcl-xL), and myeloid leukemia cell differentiation protein (Mcl-1), which are key regulators of the mitochondrial-dependent apoptotic pathway^[31,32].

To increase the therapeutic potential of TRAIL, a combination of various therapeutic agents has been examined, including bortezomib (BTZ). BTZ is a prototype proteasome inhibitor that primarily targets the $\beta 5$ subunit of the proteasome, thereby inhibiting the degradation of many proteins^[33,34]. Initially, the rationale for combining TRAIL with BTZ was based on the inhibitory effect of BTZ on I κ B degradation, consequently repressing NF- κ B activity^[35]. However, over the years, BTZ has been recognized to trigger a multitude of antiproliferative effects, all of which could contribute to increased TRAIL sensitization^[36], such as additional inhibition of the intrinsic apoptotic pathway.

In a previous study, we examined the mechanisms of acquired BTZ resistance in several NSCLC cell lines. The enhanced expression levels of the β -subunits of the proteasome, as well as point mutations, such as *Ala49Thr*, *Met45Val*, and *Cys52Phe* substitutions, within the $\beta 5$ subunit of the proteasome, were shown to contribute to BTZ resistance^[37]. In the present study, we examined the effect of TRAIL on these BTZ-resistant NSCLC cells. Interestingly, we found markedly enhanced TRAIL sensitivity in the BTZ-resistant A549 cells, whereas the parental cells were TRAIL-resistant. Both cell lines were used as a model to further investigate the molecular mechanism underlying TRAIL resistance.

METHODS

Cell culture and transfection

The human NSCLC H460, A549, and SW1573 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). H460 and A549 were grown in RPMI-1640 and SW1573 in DMEM (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Greiner Bio-One, Frinckenhausen, Germany) and 100 units/mL of penicillin/streptomycin (Lonza, Verviers, Belgium). The cells were grown at

37 °C in a humidified atmosphere of 5% CO₂ and regularly checked for mycoplasma infections.

BTZ-resistant cells were established by exposing cells to gradually increasing concentrations of BTZ over a period of at least 6 months: H460 (from 5 to 100 nM), A549 (5–40 nM) and SW1573 (5–50 nM). The resistant variants are further referred to as H460BTZR, A549BTZR, and SW1573BTZR, respectively^[37]. BTZ-resistant cells were cultured in BTZ-free medium for at least 72 h before the initiation of the experiments to exclude the interference of the selective BTZ concentrations.

For the generation of stable transfectants, H460, A549, and A549BTZR cells were transfected with 10 µg cDNA encoding Bcl-xL subcloned into the expression vector pEFLAGpGKpuro^[38] using FuGENE® 6 Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. Selection was made using increasing concentrations of puromycin (Sigma-Aldrich, Zwijndrecht, the Netherlands) ranging from 1–2 µg/mL.

Drug sensitivity was determined using the tetrazolium (MTT) assay as described earlier^[39]. Briefly, cells were plated in 96-wells plates and 24 h (day 0) after attachment drugs (BTZ or TRAIL) were added. After 72 h (day 3), the growth inhibition was measured by using the MTT assay as described. IC₅₀ values were determined by correcting optical density at day 3, by the initial optical density (OD) on the day of drug addition (day 0). The value on day 3 was set at 100% and that on day 0 at 0%. IC₅₀ values were defined as the concentration at which 50% growth inhibition was established. OD values on day 3 below that of day 0 are defined as cell kill.

Reagents and antibodies

BTZ (Velcade®) was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA, USA). RhTRAIL^[39], TRAIL-R1-specific TRAIL variant 4C7^[40], and TRAIL-R2-specific TRAIL variant D269H/E195R^[41] were produced non-commercially in cooperation with IQ-Corporation (Groningen, the Netherlands) following a protocol described earlier. The broad-spectrum caspase inhibitor zVAD-fmk was purchased from Promega, Madison, USA. For the western blot, the primary antibodies included mouse monoclonal caspase-8 (1C12), rabbit anti-caspase-9 (human specific), rabbit anti-cleaved caspase-9 (D330) (human specific), rabbit anti-caspase-3, rabbit anti-cleaved caspase-3 (Asp175), rabbit anti-PARP, rabbit anti-FLIP, rabbit anti-Bcl-2, rabbit anti-Bcl-xL, rabbit anti-Mcl-1, rabbit anti-BID, anti-caspase-1, anti-caspase-4, anti-caspase-5 (Cell Signaling Technology, Danvers, MA, USA), mouse anti-XIAP clone 2F1 (MBL International, Woburn, MA, USA), mouse monoclonal anti-NOXA, rabbit anti-TRAIL receptor 2 (Merck KGaA, Darmstadt, Germany), goat anti-DR4 (C20) (Santa Cruz Biotechnology, Heidelberg, Germany), and anti-β-actin (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands), which were diluted in InfraRedDye blocking buffer (Rockland Inc., Pottstown, PA, USA). The secondary antibodies were goat-a-mouse-InfraRedDye (1:10,000, 800CW; #926-32210 and 680; #926-32220, Westburg, Leusden, the Netherlands), goat-a-rabbit-InfraRedDye (800CW; #926-32211 and 680; #926-32221, Westburg, Leusden, the Netherlands), or donkey-a-goat-InfraRedDye (800CW; #926-32214, Westburg, Leusden, the Netherlands). Necrostatin-1, a specific RIPK1 inhibitor of necroptosis^[42], was obtained from Selleck (Houston, TX, USA).

Clonogenic survival

Clonogenic survival was performed as described previously^[43]. Briefly, cells were seeded at a density of 250 cells/well and treated with 100 ng/mL TRAIL. After 6 h exposure, the cells were washed and the medium replaced by drug-free medium, followed by 1–2 weeks incubation to allow for colony formation. At this stage, the cells were washed with PBS, fixed with 99% ethanol, and washed again. The colonies were stained with 10% Giemsa (Merck, Darmstadt, Germany), and those consisting of >50 cells were counted. The surviving fraction was determined by dividing the amount of colonies by the amount of cells plated. The surviving

fraction of untreated cells was set to 1.

Western blot analysis

Western blot analysis was performed as described previously^[37]. Briefly, the protein samples were separated by 8%-15% SDS PAGE, electroblotted onto a PVDF membrane (Millipore, Amsterdam, the Netherlands) and blocked in InfraRedDye blocking buffer. The membrane was probed with the indicated primary antibodies (overnight at 4 °C), followed by 1 h incubation with the indicated secondary antibodies. Fluorescent proteins were detected by an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA), 84 µm resolution, 0 mm offset, and with high quality.

Cell surface expression of TRAIL receptors

TRAIL receptor membrane levels were determined on BTZ-resistant cells and their parental counterparts, as described previously^[44]. Briefly, cells were collected, washed, and incubated with primary antibodies against TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 (clones HS101, HS201, HS301, and HS402, 10 µg/mL; Alexis, London, UK) for 15 min at room temperature (RT). Mouse IgG1 was used as a negative control. The cells were washed, followed by incubation with rabbit-anti-mouse PE labeled (DAKO) for 15 min at RT. The cells were washed, and the fluorescence was measured on a FACS-Calibur flow cytometer. The relative fluorescence intensity (RFI) was calculated as the mean fluorescence intensity (MFI) of the TRAIL receptor-MFI of IgG1. The basal levels were set at 100%.

RNA interference

For the transient gene knockdown, cells were seeded at 2.5×10^5 per well in a 6-well tissue culture plate and allowed to settle overnight. siRNA duplexes were formed using oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV). The culture medium was replaced with OPTI-MEM®I (Invitrogen, Breda, the Netherlands), and the cells were transfected with 100 nM small interfering RNA (siRNA) molecules targeting the sense GGAAGAGAACAGGACUGAGGC and antisense GCCUCAGUCCUGUUCUCUUC strands of *Bcl-xL* and the GAAUAGAGGCAGAUUCUGA sense and UCAGAAUCUGCCUCUAUUC antisense strands for *BID* (Eurogentech, Seraing, Belgium). As a control, siRNA with no homology to the human genome was used. After 24 h, the cells were reseeded for flow cytometric analysis.

Flow cytometric analysis of cell cycle distribution

Cell cycle analysis and cell death measurements were performed as described previously^[45]. Briefly, cells were harvested after 24 h of exposure to TRAIL and centrifuged for 5 min at 1,200 rpm (250 g). Subsequently, the cells were stained with propidium iodide buffer (0.1 mg/mL propidium iodide, 0.1% RNase A) in the dark on ice. The DNA content was analyzed by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. Cell death was determined by the sub-G₁ peak.

Fractionation lipid rafts

Fractionation of the lipid rafts from the non-lipid rafts was performed as described previously^[23]. Briefly, 1×10^8 cells were lysed on ice in 2 mL MNX buffer [1% Triton X-100 in 25 mM MES and 150 mM NaCl (pH 6.5)] supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) and then homogenized. The homogenates were resuspended in 2 mL 90% sucrose dissolved in MNX buffer and gently overlaid with 4 mL 35% sucrose and 4 mL 5% sucrose. The samples were centrifuged at $175,000 \times g$ in a SW32Ti rotor with an Optima L-80 XP centrifuge (Beckman, Brea, CA, USA) for 16 h at 4 °C. Ten fractions of 1 mL were collected from top to bottom of the gradient and analyzed by western blot.

mRNA expression of apoptosis specific genes by RT-qPCR

To detect alterations in the mRNA expression levels of several pro- and anti-apoptotic genes, we performed an RT² Profiler PCR Array (SABiosciences, Frederick, MD, USA), which was capable of detecting 84 apoptotic genes with qPCR according to the manufacturer's instructions. Briefly, the RNA was isolated using the RNeasy 96 kit (Qiagen Inc., Valencia, CA, USA). The cDNA was prepared with the RT² First strand kit, mixed with the RT²SYBR Green qPCR master mix, and aliquoted across the PCR array. Amplification data (C_t values) were collected with an ABI 7500 RT-PCR system. $\Delta\Delta C_t$ was determined as $(A549BTZRC_t - A549BTZRC_{t \text{ housekeeping genes}}) - (A549C_t - A549C_{t \text{ housekeeping genes}})$. The fold change was calculated by $2^{\Delta\Delta C_t}$, indicating the upregulation of genes with a fold change > 1 in the A549BTZR cells and the downregulation of genes with a fold change < 1 .

Detection of human osteoprotegerin by ELISA

The levels of secreted osteoprotegerin (OPG) were detected using a RayBio® Human Osteoprotegerin ELISA Kit according to the manufacturer's instructions (RayBiotech, Inc., Norcross, GA, USA). Briefly, supernatants of different cell lines were collected, centrifuged for 5 min at 1,500 rpm (300× g) and sampled on an osteoprotegerin microplate coated with anti-human osteoprotegerin. After incubation for 2.5 h at RT, the wells were washed and incubated with biotinylated antibody for 1 h at RT. The solution was discarded, and after washing, streptavidin solution was added for 45 min at RT. After washing, TMB One-Step Substrate Reagent was added for 30 min at RT. Stop solution was added, and the optical density was measured at 450 nm.

Secretion of cytokines

A549 and A549BTZR cells were plated in 6-well plates at a density of 3×10^5 cells. After 24 h, the supernatant was collected and centrifuged for 5 min at 1,500 rpm to remove detached cells. According to the manufacturer's protocol, the cytometric bead array (CBA) human inflammatory cytokines kit (BD biosciences, San Jose, CA, USA) was used for the quantitative measurement of interleukin-8 (IL-8), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), TNF, and interleukin-12p70 (IL-12p70). Measurements were performed on a FACS-Calibur flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) using the CellQuest program.

Statistics

Statistical evaluation was performed using the Student t-test, with $P < 0.05$ as the level of significance, unless otherwise indicated.

RESULTS

Phenotypic alteration for TRAIL sensitivity in A549BTZR cells

Previously, we generated acquired BTZ resistance in a panel of NSCLC cells that displayed different TRAIL sensitivities^[18,37]. We used H460 (IC₅₀: 6 ng/mL TRAIL), A549, and SW1573 cells (IC₅₀: > 200 ng/mL TRAIL). Since BTZ is a potent sensitizer for TRAIL-induced apoptosis, we examined TRAIL sensitivity in the generated acquired BTZ-resistant cell lines. Using the MTT assay for determination of initial sensitivity, the IC₅₀ for TRAIL in H460BTZR cells slightly increased to 9.9 ng/ml, did not change in SWS1573BTZR cells (> 200 ng/mL), but decreased in A549BTZR cells (11.3 ng/mL). However, at a higher concentration (100 ng/mL), we found that in A549BTZR and H460BTZR cells, a higher extent of cell kill was observed compared to A549 and H460 cells. Based on the MTT assay data, we chose one concentration of TRAIL (100 ng/mL) to be tested in clonogenic assays since this assay gives a better estimate of surviving fractions. In clonogenic assays, TRAIL sensitivity was slightly increased in H460BTZR compared to parental H460 cells [Figure 1A]. Interestingly, also with the clonogenic assay, the A549BTZR cells were significantly more sensitive to TRAIL-induced apoptosis, as shown by the strong decline in the clonogenic outgrowth with

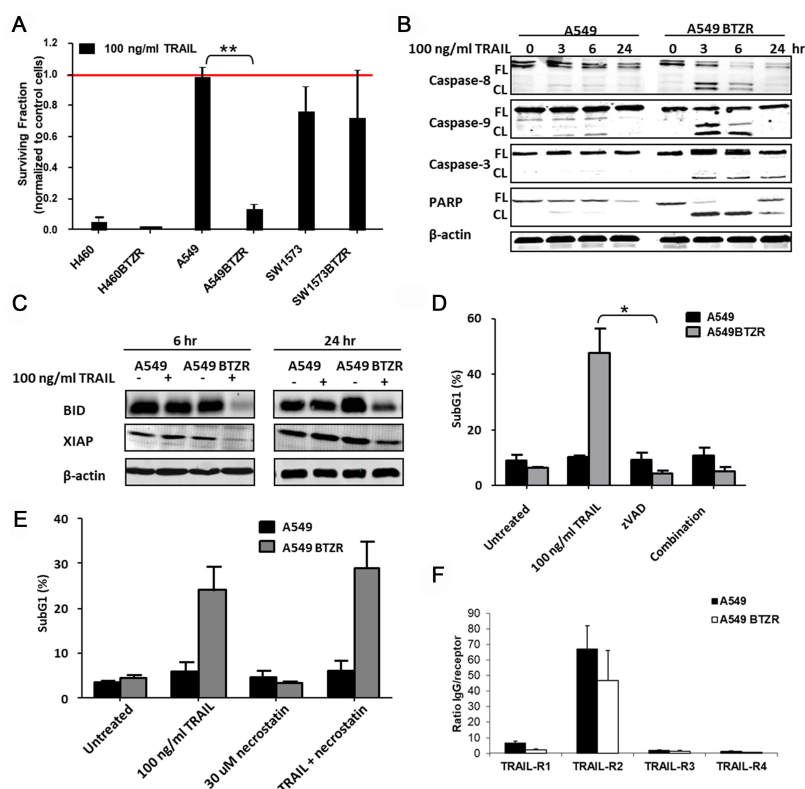


Figure 1. Acquired BTZ-resistant A549 cells demonstrate reversed phenotypic TRAIL sensitivity. (A) The clonogenic outgrowth was significantly reduced in both the H460BTZR (not measurable) and A549BTZR cells after exposure to 100 ng/mL TRAIL for 6 h; (B) Western blot analysis of caspase and PARP cleavage shows the enhanced cleavage already after 6 h in the A549BTZR cells; (C) BID and XIAP were reduced in the A549BTZR cells after exposure to 100 ng/mL TRAIL for 6 and 24 h; (D) the zVAD (100 μ g/mL) completely reduced the TRAIL-enhanced apoptosis after 24 h exposure as determined with Flow Cytometry; (E) Incubation with 30 μ M necrostatin for 24 h did not result in reduced TRAIL-induced apoptosis as determined with Flow Cytometry; (F) Expression of TRAIL in A549BTZR cells as determined with Flow cytometry. Similarly, the TRAIL expression was not different between parental and resistant variants of H460 and SW1573 cells. The values are the means $\pm$ SEM and the western blots depicted represent at least three independent experiments. Similarly, the effect of TRAIL was significantly higher in A549BTZR compared to A549 cells. $^{**}P < 0.001$; $^{*}P < 0.01$. BTZ: Bortezomib; TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor; BID: BH3 Interacting Domain Death Agonist; XIAP: X-linked inhibitor of apoptosis protein.

surviving fractions of 0.98 to 0.13 for the A549 and A549BTZR cells, respectively ($P < 0.005$). Therefore, for further mechanistic studies, we focused on the A549 cells and their BTZ-resistant variant A549 BTZR.

In order to determine whether increased apoptosis was responsible for this collateral sensitivity, we analyzed time-dependent caspase cleavage after exposure to TRAIL using western blot analysis. TRAIL increased levels of caspase-8, caspase-9, caspase-3, and PARP cleavage in the A549BTZR cells compared to the parental cells [Figure 1B]. In addition, both BID and XIAP were cleaved after 6 h of exposure to TRAIL in the A549BTZR cells, whereas no cleavage was detected in the A549 cells up to 24 h of treatment [Figure 1C]. To determine whether the increased TRAIL-induced cell death was caspase-dependent, we investigated the effect of zVAD, a broad-spectrum caspase inhibitor, on TRAIL-induced apoptosis [Figure 1D]. After 24 h of exposure, the cell death was significantly reduced ($P < 0.01$) when exposed to both TRAIL and zVAD, indicating that the TRAIL-induced apoptosis in the A549BTZR cells was caspase-dependent. The exposure of cells to 30 μ M necrostatin in combination with TRAIL did not reduce the amount of cell death in A549BTZR, thereby excluding the activation of cell death via necroptosis [Figure 1E]. TRAIL expression was similar between parental and resistant cells [Figure 1F].

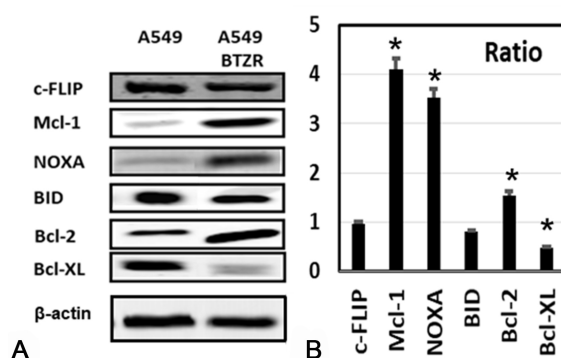


Figure 2. Basal protein levels were differently expressed in the A549 and A549BTZR cells. (A) Western blot analysis was used to detect the basal expression levels of several proteins in the A549 and A549BTZR cells. The blots are representative of at least three separate experiments. β -actin was used as a loading control; (B) Scans showing the ratio A549BTZR/A549. * $P < 0.01$.

mRNA and protein levels of apoptotic proteins are differentially expressed in A549BTZR cells

The enhanced TRAIL sensitivity in the A549 BTZ-resistant variant provides a very interesting model to further characterize the underlying mechanisms that regulate TRAIL-induced apoptosis. Therefore, we determined the basal mRNA expression levels of 84 apoptosis-related genes in A549 and A549BTZR cells using an RT²ProfilerTM PCR array. The top ten genes that were overexpressed or downregulated in A549BTZR are shown in Table 1. In line with the alterations found with the RT²ProfilerTM PCR array, we observed that the protein expression levels of c-FLIP and BID were not differentially expressed in A549 and the BTZ-resistant counterpart [Figure 2]. Moreover, the expression level of Bcl-xL was strongly reduced in the A549BTZR cells, whereas the expression of Bcl-2 and Mcl-1 increased. Although Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) was not included in the RT-PCR array, we also evaluated the protein levels of this Mcl-1 inhibitor and found that NOXA was also overexpressed in the A549BTZR cells. The relationships among the downregulated and upregulated apoptotic genes were further analyzed by an IPA pathway analysis [Table 2], and schematic figures of the network functions are represented in Supplementary Figure 1. Cell death, cell-mediated immune response, and cellular development were predominantly found as associated network functions. Taken together, these findings indicate that a broad range of pathways relevant to cell survival are altered in A549BTZR cells.

A549BTZR cells secrete high levels of osteoprotegerin

The *TNFRSF11B* mRNA, encoding the soluble TRAIL receptor, OPG, was the highest upregulated gene in A549BTZR cells. This finding is remarkable since OPG is thought to inhibit the apoptosis-inducing effect of TRAIL. An ELISA analysis showed that the secretion of OPG in the A549BTZR cells strongly increased by 122-fold, from 3 pg/mL to 364 pg/mL OPG for the A549 and A549BTZR cells, respectively [Figure 3A]. Notably, the TRAIL-sensitive H460 cells also had a high level of OPG secretion, but were very low in both SW1573 and SW1573BTZR cells. However, the incubation of the TRAIL-resistant A549 or SW1573 cells with 900 pg/mL OPG for 4 h prior to the TRAIL treatment did not affect cell death activation [Figure 3B]. Thus, the increases in OPG secretion could not be linked with the increased TRAIL sensitivity in the A549BTZR cells.

Altered secretion of cytokines does not influence TRAIL sensitivity

The mRNAs for *caspase-1*, *caspase-4*, and *caspase-5* also strongly increased in the BTZ-resistant cells [Table 1]. These caspases are involved in the regulation of inflammatory responses [Supplementary Figure 1]^[46]. A western blot analysis showed an increased expression of caspase-1 and caspase-4 but not of caspase-5 [Figure 4A]. Since these caspases are part of the inflammasome and play a role in the immune system by promoting the maturation and secretion of pro-inflammatory cytokines, we examined the cytokine secretion of A549

Table 1. A549BTZR cells display an altered mRNA expression pattern

	Fold Change
A: Gene	
<i>TNFRSF11B</i>	75
<i>CASP5</i>	28
<i>CASP4</i>	21
<i>CASP1</i>	10
<i>CD70</i>	8
<i>MCL1</i>	4
<i>BCL2</i>	4
<i>TNFRSF25</i>	3
<i>CASP3</i>	3
<i>GADD45A</i>	2
B: Gene	
<i>DAPK1</i>	0.0006
<i>PYCARD</i>	0.0206
<i>TNFSF8</i>	0.0304
<i>BCL2A1</i>	0.0311
<i>TNFRSF9</i>	0.0404
<i>TNFRSF21</i>	0.0519
<i>BCL2L1</i>	0.0644
<i>TP53</i>	0.0850
<i>BAD</i>	0.1325
<i>BFAR</i>	0.1554

RT-qPCR analysis resulted in a top 10 of mRNAs that were expressed higher (A) or lower (B) in A549BTZR cells. Values were corrected for the average of five different housekeeping genes. Values found for A549 cells were set to 1 and values of A549BTZR cells were correlated to determine the fold change.

Table 2. Network functions associated with the altered mRNA expression levels in the A549BTZR cells

ID	Associated network functions	Score
1	Cell death, embryonic development, renal necrosis/cell death	49
2	Cell death, cell-mediated immune response, cellular development	41
3	Cell death, cell-mediated immune response, cellular development	32
4	Cell death, dermatological diseases and conditions, genetic disorder	15
5	Cell death, cell cycle, antimicrobial response	13

An analysis using IPA resulted in a list of the top 5 networks associated with the modulated mRNA expression levels in the A549BTZR cells compared to the A549 cells.

and A549BTZR cells. IL-8 and IL-6 levels were found to be increased by 11- and 103-fold, respectively, whereas the secretion of IL-1 β , IL-10, TNF- α , and IL-12 p70 was reduced [Figure 4B].

Since several alterations in cytokine secretion were found between TRAIL-sensitive and -resistant cells, we investigated whether TRAIL sensitivity is affected by a combination of secreted factors and not just by one factor such as OPG. Therefore, we cultured A549 cells in conditioned medium collected from TRAIL-sensitive H460 or A549BTZR cells and exposed the cells to 100 ng/mL TRAIL. However, the apoptosis-inducing effect of TRAIL did not change [Figure 4C]. The opposite was also examined by

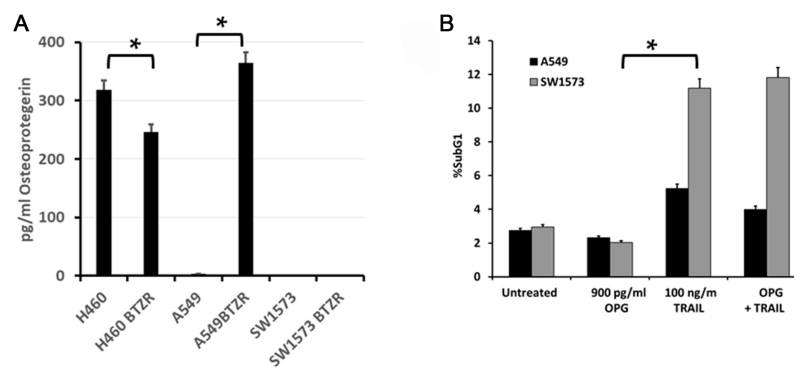


Figure 3. Enhanced secretion of OPG by A549BTZR did not induce TRAIL sensitivity. (A) With the use of ELISA, the secreted OPG levels were measured in the supernatant of the different cell lines; *significantly different ($P < 0.01$). The secretion of OPG by both SW1573 variants was 0.41 pg/mL; (B) Cell death was measured in the TRAIL-resistant A549 and SW1573 cells after incubation with 900 pg/mL OPG for 4 h followed by the addition of 100 ng/mL TRAIL for 24 h. The effect of TRAIL was significantly higher than that of OPG ($P < 0.01$). The values are the means of at least three independent experiments $\pm$ SEM. OPG: Osteoprotegerin; TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor.

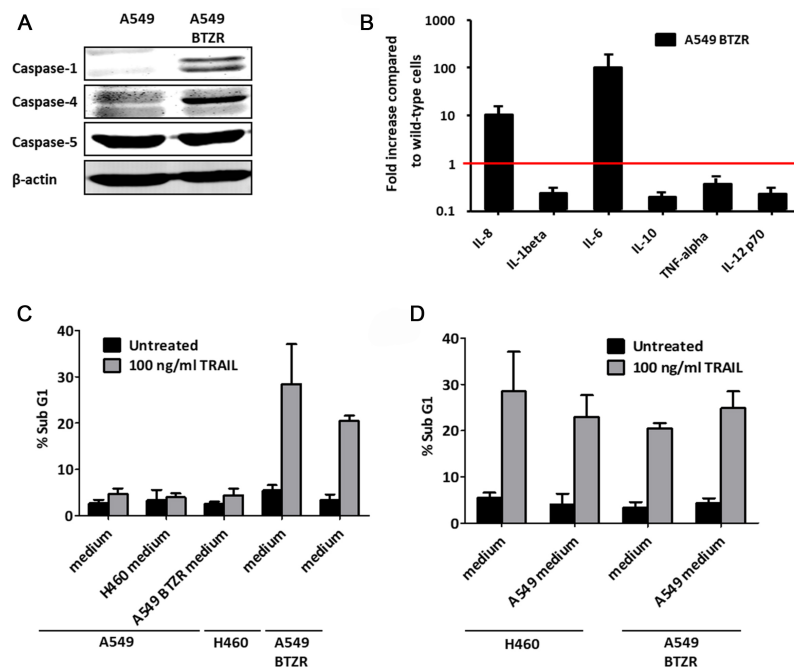


Figure 4. Different secretion products from the A549BTZR cells did not trigger TRAIL sensitivity. (A) Western blot analysis was used to detect the basal expression levels of caspase-1, -4 and -5 in A549 and A549BTZR cells; (B) Supernatant of the A549 and A549BTZR cells was used to determine the cytokine secretion. The cytokine secretion levels detected in the A549BTZR supernatant were compared with the secretion by A549 cells, which was set to 1 (calculated from the means); (C) Cell death (subG1) was measured in A549 cells exposed to 100 ng/mL TRAIL for 24 h in supernatant collected from the H460 and A549BTZR cells. As a control, H460 and A549BTZR cells were exposed to 100 ng/mL TRAIL for 24 h in fresh medium; (D) Cell death (SubG1) in the H460 and SW1573 cells was determined after exposure to 100 ng/mL TRAIL for 24 h in culture medium or supernatant collected from A549 cells. The values are the means $\pm$ SEM of at least three independent experiments, while the blots are representative of at least 3 separate experiments. The effect of TRAIL treatment on H460 and A549BTZR cells on induction of apoptosis (C and D) was significant ($P < 0.001$). TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor.

incubating H460 and A549BTZR cells with A549-conditioned medium with or without 100 ng/mL TRAIL. However, the conditioned A549 medium also did not affect TRAIL sensitivity in H460 and A549BTZR cells [Figure 4D]. Taken together, these results indicate that although the secretion of OPG and other cytokines is different in TRAIL-sensitive A549BTZR cells compared to parental cells, this does not affect TRAIL sensitivity.

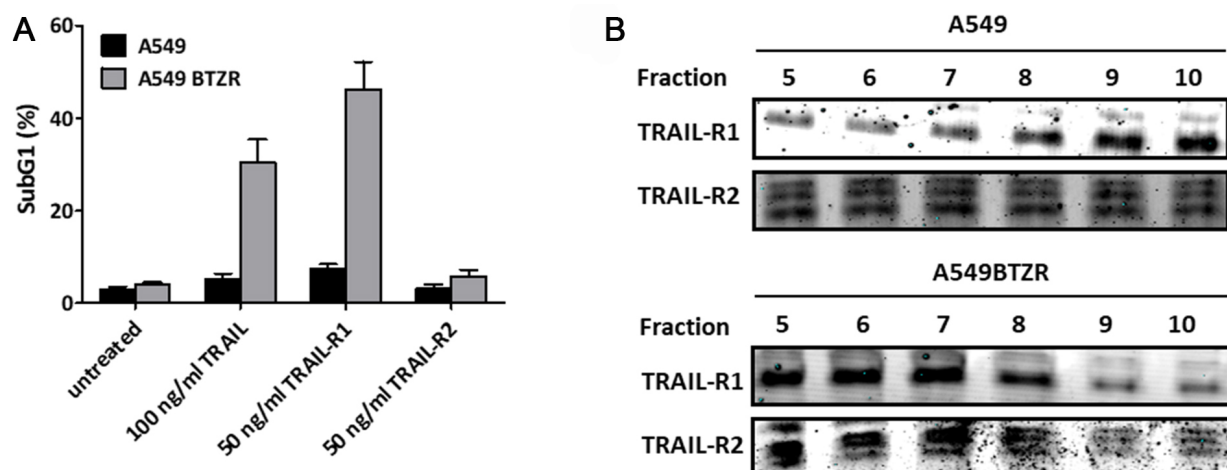


Figure 5. A549BTZR TRAIL-induced apoptosis was mediated via TRAIL-R1, which was redistributed into lipid rafts. (A) Cell death was determined in the A549 and A549BTZR cells after exposure to 100 ng/mL TRAIL, 50 ng/mL 4C7 (TRAIL-R1-specific TRAIL variant), or 50 ng/mL D269HE195R (TRAIL-R2-specific TRAIL variant) for 24 h. The effect of TRAIL and TRAIL-R1 was significant in A549BTZR cells compared to A549 parental ($P < 0.0001$); (B) Western blot analysis of the TRAIL-R1 and -R2 expression levels in different cellular fractions, with fractions 5-7 and fractions 8-10 representing the lipid and non-lipid rafts, respectively. The values are the means $\pm$ SEM of at least three independent experiments, while blots are also representative of at least 3 separate experiments. TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor.

TRAIL-R1 mediates TRAIL-induced apoptosis in A549 BTZ-resistant cells, and its localization is altered to the lipid rafts

To further examine the underlying mechanism of TRAIL sensitization, we determined which of the TRAIL receptors mediates TRAIL-induced apoptosis. A549BTZR and A549 cells were exposed to TRAIL-receptor-targeting TRAIL-variants-targeting antibodies that were either specific for TRAIL-R1 (4C7) or TRAIL-R2 (D269H/E195R). Strong induction of cell death was detected when the A549BTZR cells were exposed to 50 ng/mL TRAIL-R1 antibody, whereas no cell death was found after exposure to the TRAIL-R2-specific antibody [Figure 5A], indicating a pivotal role for TRAIL-R1. TRAIL receptor levels are often upregulated upon BTZ treatment as a mechanism to sensitize cells for TRAIL-induced apoptosis^[6]. However, the mRNA levels were not significantly altered in the A549BTZR cells. In line with this, a FACS analysis showed no significant difference in the TRAIL receptor expression levels at the cell membrane between the parental A549 and A549BTZR cells [Figure 5B]. Though the TRAIL receptor expression levels were not altered, the redistribution of the TRAIL receptors into the lipid rafts may be of great importance for inducing apoptosis^[26]. Using a discontinuous sucrose density gradient, we isolated lipid rafts, which were found in fractions 5-7, whereas the non-lipid rafts were found in fractions 8-10. In the parental A549 cells, TRAIL-R1 was mainly localized in the non-lipid rafts, while TRAIL-R2 was distributed in both the lipid and non-lipid rafts [Figure 5B]. The A549BTZR cells demonstrated a clear shift of TRAIL-R1 into the lipid rafts compared to the TRAIL-R1 localization in A549. Together, this finding links the lipid raft localization of TRAIL-R1 with the observed TRAIL-R1 sensitivity in the A549BTZR cells and may be mainly responsible for the reversed TRAIL sensitivity in these cells.

TRAIL-induced apoptosis in A549BTZR cells is partially regulated by the intrinsic pathway, independent of Bcl-xL

Other mechanisms that could be involved in the reversed TRAIL sensitivity in A549BTZR cells are differences in the expression of Bcl-2 family member proteins. Interestingly, whereas the Mcl-1 and Bcl-2 expressions increased in the A549BTZR cells [Figure 2], the expression of Bcl-xL was markedly reduced. In

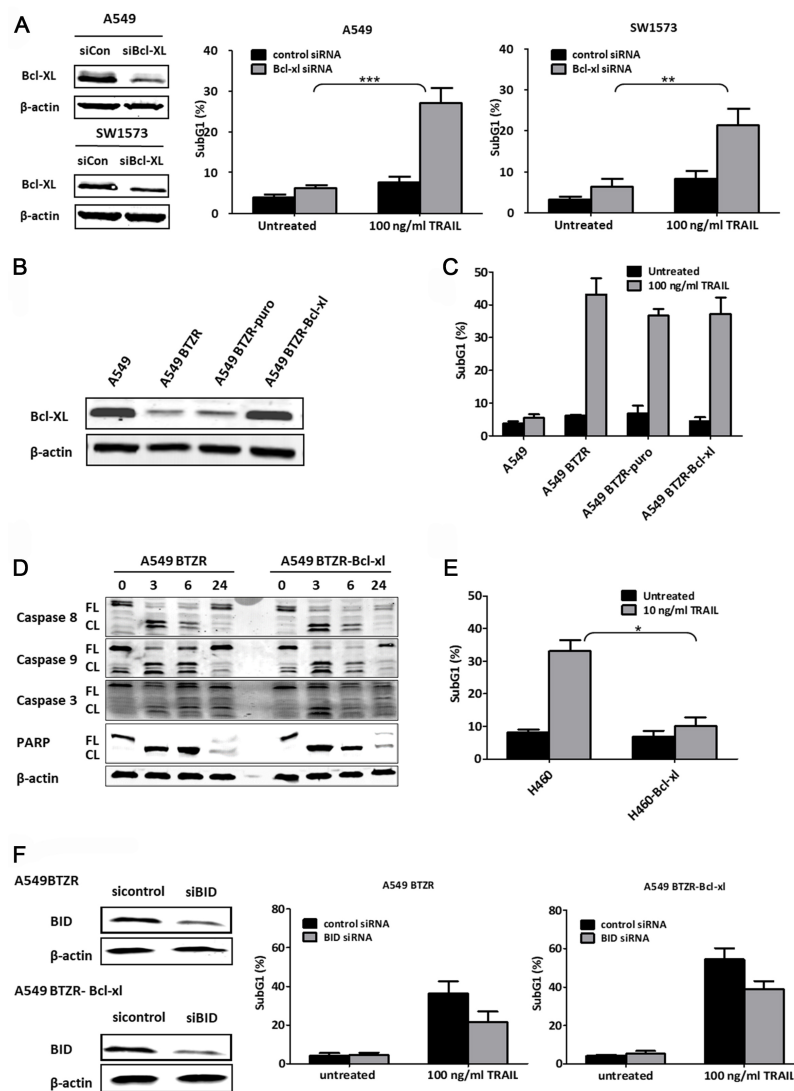


Figure 6. The intrinsic apoptotic pathway was involved in the TRAIL-induced apoptosis in the A549BTZR cells, though reduced levels of Bcl-xL were not a determinant. (A) The silencing of Bcl-xL, as shown by western blot analysis, resulted in enhanced cell death in the A549 and SW1573 cells after 24 h exposure to 100 ng/mL TRAIL; (B) A549BTZR cells were transfected with a control puromycin vector or a Bcl-xL-containing vector, as shown by western blot analysis; (C) Cell death was measured in the A549BTZR cells and the transfected variants after incubation with 100 ng/mL TRAIL for 24 h; (D) Western blot analysis was used to detect the cleavage of caspase-8, -9, -3, and PARP after exposure to 100 ng/mL TRAIL for several time intervals in the A549BTZR and A549BTZR-Bcl-xL cells; (E) Cell death was determined in the H460 and H460-Bcl-xL cells after exposure to 10 ng/mL TRAIL for 24 h; (F) Cell death was measured in A549BTZR and A549BTZR-Bcl-xL cells after silencing of BID. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor; Bcl-xL: B-cell lymphoma-extra large; BID: BH3 Interacting Domain Death Agonist.

order to investigate the role of Bcl-xL and TRAIL sensitivity in parental A549 and SW1573 cells, we silenced Bcl-xL protein expression [Figure 6A]. In both the TRAIL-treated A549 and SW1573 cells, a significant increase in cell death was detected, from 7% up to 27% ($P < 0.001$) and 22% ($P < 0.01$), respectively. We next examined whether the overexpression of Bcl-xL in the A549BTZR cells would regain TRAIL sensitivity by stably transfecting the A549BTZR cells with a vector encoding Bcl-xL to yield A549BTZR-Bcl-xL cells and control A549BTZR-puro cells. A western blot analysis confirmed the increased expression of Bcl-xL in the A549BTZR-Bcl-xL cells compared to the control vector-transfected cells [Figure 6B]. However, the sensitivity of the A549BTZR-Bcl-xL cells to 100 ng/mL TRAIL was not altered [Figure 6C]. Caspase cleavage was determined by western blot analysis, showing the cleavage of caspase-8, caspase-9, caspase-3, and PARP in both cell lines after 3 h of 100 ng/mL TRAIL exposure [Figure 6D]. To validate the functioning of the Bcl-xL encoding vector, we also transfected TRAIL-sensitive H460 cells. Exposure to 10 ng/mL TRAIL strongly reduced cell death in these cells, from 33% to 10% ($P < 0.05$), as expected [Figure 6E].

To further explore the requirement of the intrinsic apoptotic pathway for TRAIL-induced apoptosis in A549BTZR and A549BTZR-Bcl-xL cells, we silenced BID expression and observed the partial inhibition of cell death from 36% to 22% and 54% to 39% after exposure to 100 ng/mL TRAIL in the A549BTZR and A549BTZR-Bcl-xL cells, respectively [Figure 6F].

Taken together, these results indicate that both the extrinsic and intrinsic apoptotic pathways are involved in TRAIL-induced apoptosis in A549BTZR cells. Although the intrinsic apoptotic pathway in A549BTZR cells is apparently not regulated by Bcl-xL, Bcl-2 and Mcl-1, as well as other Bcl-2 family members not yet examined, might confer the phenotypic change of TRAIL sensitivity in A549BTZR cells.

DISCUSSION

Intrinsic and acquired resistance are the main hurdles in the development of effective cancer therapies. Unfortunately, many cancer cell lines and tumors are intrinsically resistant to TRAIL^[18]. In this study, we report an enhanced TRAIL sensitivity in acquired BTZ-resistant A549 cells, whereas the parental cells displayed a highly resistant TRAIL phenotype. This provided us with a very interesting model to further investigate the underlying mechanisms of TRAIL resistance.

In our study, we found a striking increase in both the OPG mRNA and protein expressions. OPG is known to be a soluble decoy receptor with a lower affinity towards TRAIL compared to TRAIL-R1 and -R2, and in contrast to these receptors, it does not transduce an apoptotic signal^[47-49]. In ovarian cancer, OPG not only attenuated TRAIL-induced apoptosis via a TRAIL-binding manner, but also stimulated the activation of pro-survival Protein kinase B (Akt) signaling via integrin/FAK signaling and inhibited TRAIL-induced apoptosis^[50]. Higher OPG expression and secretion were found in the TRAIL-sensitive H460 and A549BTZR cells compared to the resistant A549 and SW1573 cells. In addition, exogenously added OPG was not sensitive to TRAIL-induced apoptosis in the A549 and SW1573 cells. This might indicate an alternative function of OPG in addition to its role in suppressing TRAIL-induced apoptosis.

Furthermore, RT-PCR and western blot analyses of the A549BTZR cells demonstrated an increased expression of caspase-1 and caspase-4, which are considered inflammatory caspases^[51]. Caspase-1 was first identified as an IL-1 β -converting enzyme, also capable of processing IL-18, and is now classified as a cytokine processor caspase, along with caspases-4, -5 and -12^[52]. Although caspase-1 was described to activate IL-1 β , secretion of this cytokine was strongly reduced in the A549BTZR cells, whereas secretion of IL-6 and IL-8 was evidently increased. Enhanced levels of IL-6 were described to contribute to Mcl-1 upregulation and, subsequently, TRAIL resistance^[53]. Controversially, increased levels of Mcl-1 did not trigger TRAIL resistance in the A549BTZR cells. Trametinib-induced FBW7-dependent Mcl-1 ubiquitination and degradation enhanced apoptosis in colorectal cancer cell lines^[54], while Tolksdorf et al. showed that Mcl-1 silencing may overcome the resistance of melanoma cells against TRAIL-armed oncolytic adenovirus^[55]. However, our study showed that this effect is not very strong in NSCLC cells. Furthermore, the increased secretion of IL-6 and IL-8 was not sufficient to regulate TRAIL receptor and c-FLIP expression levels. This is in contrast to previous studies where IL-6 and IL-8 have been reported to increase c-FLIP expression levels and IL-8 was shown to reduce TRAIL-R1 expression levels, resulting in decreased TRAIL sensitivity^[56-58]. These data indicate a complex tumor-cell-dependent interaction between TRAIL and interleukins, which may be different in NSCLC cells and needs to be investigated more in depth. Previous studies revealed that TRAIL by itself can increase levels of IL-8 via caspase-1 and -8 activation, thereby attenuating cell death^[59,60], or via TRAIL-R1 (which is enhanced in the presence of TRAF2 and Bcl-xL)^[61]. In addition, BTZ increased the IL-8 level in ovarian cancer cells^[62] and in human monocytes and macrophages *in vitro*, thereby also inhibiting the expression of IL-6 and IL-1 β ^[63], which may explain why its expression was increased in our study. A similar observation was made in prostate cancer cell lines in which BTZ treatment significantly

increased IL-8 levels mediated by I κ B kinase (IKK) α . The inhibition of this kinase enhanced the BTZ-induced p65 recruitment to the IL-8 promoter and subsequently increased IL-8 expression^[64]. Similar findings were noted also in ovarian cancer cell lines^[65]. The decrease in the IL-10 level in our study may also be stimulated by BTZ, as it was previously found in cutaneous T-cell lymphoma cells^[66]. Another finding concerning BTZ is that it increased caspase-8, -9 and -3 activities in head and neck cancer cells and in gastric cancer cells^[67,68]. Interestingly, the increase in caspase-3 may be mediated by the interaction with transmembrane and tetratricopeptide repeat-containing 2 (TMTC2) in the lipid raft domains. This molecule, enhancing the response for TRAIL, may be considered a potential predictive biomarker^[69].

Exploring the role of secreted cytokines on TRAIL-induced apoptosis demonstrated that the A549 parental cells did not secrete any factors to inhibit TRAIL-induced apoptosis. In addition, increased levels of OPG, IL-8, and IL-6 were not adequate to enhance TRAIL-induced apoptosis. Therefore, the inflammatory caspases might comprise an alternative role in TRAIL-induced apoptosis, since it has been described that the activation of caspase-4 via TRAIL-induced ER stress is required for enhanced caspase-3 activation^[70]. Therefore, the contribution of ER stress in A549BTZR cells needs further exploration. Currently, more functions of caspase-1 include the activation of NF- κ B^[71] and induction of pyroptosis, a pathway of programmed cell death distinct from apoptosis, which occurs independently of caspase-8 and -3^[72]. However, due to the high levels of both caspase-8 and -3 cleavage, it is unlikely that A549BTZR cells are subjected to pyroptosis, while another study showed that TRAIL induced only apoptosis but not necroptosis, supporting the important role of apoptotic mechanisms in TRAIL sensitivity^[73]. Caspase-1 is also involved in the TRAIL signaling pathway, since specific caspase-1 inhibitors abrogated TRAIL-induced apoptosis^[74-76]. In addition, caspase-1 was previously shown to be involved in the induction of TRAIL-R2-mediated cell death in glioma cells^[59]. Moreover, the pro-domain of caspase-1 is translocated into the nucleus to enhance Fas-mediated death signals, most likely through enhanced caspase-8 activation or reduced nuclear NF- κ B activation^[77]. Whether caspase-1 is also involved in TRAIL-R1 signaling in NSCLC cancer cells is unknown; however, enhanced (pro)caspase-1 might play a role in TRAIL-induced apoptosis in A549BTZR cells.

The trimerization of the TRAIL receptors on the cell surface, followed by the assembly of DISC and caspase-8 cleavage, is the initial step for TRAIL-induced apoptosis via the extrinsic pathway. Together with the silencing of BID, the evident cleavage of caspase-8 in A549BTZR cells further emphasizes the role of TRAIL receptors and DISC assembly. For several cell lines, including NSCLC cells and in our study, the cytotoxic effect of TRAIL receptors was most efficient when distributed in lipid rafts^[22,23,78], while we also demonstrated that TRAIL sensitivity was particularly induced via TRAIL-R1, which was redistributed towards the lipid rafts, similar to gastric cancer^[68]. These findings indicate a correlation between TRAIL receptor membrane distribution and sensitivity. Interestingly, in head and neck cancer, the major player was not TRAIL-R1 but TRAIL-R2^[67]. Based on the literature, both receptors have no significant structural differences, and in various neoplasms, the dominant mechanism of apoptosis induction may be more related to TRAIL-R1, whereas in others, instead to TRAIL-R2. There are hypotheses that the S-palmitoylation site present in TRAIL-R1 (but not in TRAIL-R2) or the GXXXG motif present in TRAIL-R2 (but not in TRAIL-R1) may be determining factors^[79,80]. However, appropriate studies should be conducted for specific neoplasms in the future.

Upon recruitment of the DISC, pro-caspase-8 can be activated. However, some studies suggest that the dimerization and autocleavage of caspase-8 are not sufficient for full activation^[81,82]. Active caspase-8 has been proposed to be very unstable^[82,83]. The polyubiquitination of caspase-8 by the Cullin-3 (CUL3) E3 ligase has been shown to maintain the stability of caspase-8. Upon polyubiquitination of caspase-8, p62 is recruited to the DISC, followed by the translocation of the DISC, including the ubiquitinated caspase-8 and p62, into the cytosol to form an aggresome, which will induce cell death^[81,84]. However, whether p62 or CUL3 is

involved in the increase in TRAIL-induced apoptosis in A549BTZR cells is not yet clear. Interestingly, Huang *et al.* showed that caspase-8 cleaves BID to its truncated form, tBID^[85]. However, in a tBID mutant knockout for the mitochondrial targeting helices ($\alpha 6$ and $\alpha 7$), apoptosis significantly decreased, indicating a crucial role of the outer mitochondrial membrane in TRAIL sensitivity. Moreover, a mutation in caspase-8 decreased TRAIL-induced apoptosis^[86]. These data are in agreement with the predictive role of IL-8, IL-10, BID, and TNF- α in TRAIL treatment^[87,88]. Caspase-8 may also cause TRAIL-mediated STAT1 phosphorylation and the induction of IFN-related genes^[89].

In addition to the trimerization of TRAIL receptors in lipid rafts, TRAIL receptors can also trimerize when distributed in non-lipid rafts. This is often correlated to the activation of the secondary complex consisting of FADD and RIP, which initiates cell survival via the activation of NF- κ B. Moreover, an enhancement of the NF- κ B activity upon TRAIL exposure has been described for A549 cells^[90,91]. Therefore, one might speculate that enhanced TRAIL sensitivity in A549BTZR cells was promoted by increased levels of caspase-8 activation in combination with reduced NF- κ B activity. However, NF- κ B activity induced by TRAIL was also found in TRAIL-sensitive H460 cells^[32]. In addition, Yang *et al.* described that resistance to the combination of TRAIL and BTZ in A549 spheroid models was Bcl-2-dependent and not induced by NF- κ B activity^[92], thereby questioning the relevance of NF- κ B activity in TRAIL resistance. However, in ovarian cancer, it was demonstrated that the inhibition of the NF- κ B (p65) pathway via resveratrol significantly increased the efficiency of TRAIL-induced apoptosis in NSCLC, while pro-survival effects by NF- κ B, Akt, and ERK(1/2) and anti-apoptosis actions by Six1 inhibited the TRAIL-R1/TRAIL-R2 pathways^[93,94]. Moreover, NF- κ B-regulated microRNAs stimulated resistance to TRAIL^[95]. CXCL1, a target for NF- κ B, stimulated immune cell infiltration via paracrine signaling, leading to TRAIL resistance in PDAC^[96]. Based on these results, it can be concluded that NF- κ B may influence cellular behavior via various signaling pathways, which should be further investigated.

Bcl-2 family members have already been identified to hamper TRAIL-induced apoptosis via the intrinsic apoptotic pathway^[38,92]. Knockdown experiments of BID revealed that TRAIL-induced apoptosis in A549BTZR cells was also partially regulated by the intrinsic pathway and not only exerted via the extrinsic apoptotic pathway.

We further explored the role of reduced Bcl-xL expression in TRAIL sensitivity. However, although silencing of Bcl-xL sensitizes parental A549 for TRAIL-induced apoptosis, low basal levels of Bcl-xL in A549BTZR cells were not responsible for enhanced TRAIL sensitivity. The importance of Bcl-xL in TRAIL resistance appears to be variable in different cancer types (NSCLC, pancreatic cancer, glioma) and depends on the apoptotic trigger(s) applied^[93,97-100]. In addition to Bcl-xL, other Bcl-2 family members were also differentially expressed in A549BTZR cells when compared to A549 cells. Increased levels of Bcl-2 and Mcl-1 indicate enhanced TRAIL resistance via the intrinsic apoptotic pathway. Nevertheless, NOXA might counteract enhanced Mcl-1 protein levels, e.g., by relocalization of Mcl-1 to mitochondria, an effect which needs further investigation^[101]. In addition to NOXA, TRAIL-induced activation of p38 and JNK may also counteract the effect of Mcl-1 expression^[102]. The mRNA levels of the pro-survival protein Bcl-2-A1 were reduced in A549BTZR cells, which may stimulate proapoptotic signaling and sensitivity to TRAIL^[103]. Therefore, the differences identified in the expression of proteins active in the intrinsic apoptotic pathway together might tip the balance towards TRAIL-induced apoptosis.

Taken together, an abundant number of alterations were found in the A549BTZR compared to the A549 parental cells. A limitation of the study is that these aberrations were found in cells made resistant to BTZ and were not found in all resistant variants. These alterations might indicate increased levels of TRAIL resistance, such as increased Mcl-1 and Bcl-2 expression levels and enhanced secretion of OPG, IL-6, and

IL-8. Nonetheless, A549BTZR cells show a TRAIL-sensitive phenotype. Therefore, the combination of several modulations contributes to TRAIL sensitivity, such as the (1) involvement of the immune system, with the enhanced activation of caspase-1 and -4; (2) activation of the extrinsic apoptotic pathway, which might be caused by TRAIL-R1 redistribution into lipid rafts; and (3) triggering of the intrinsic apoptotic pathway via alterations in the expression levels of multiple Bcl-2 family members. Further clarification regarding these mechanisms of action would provide better insight into TRAIL sensitivity and might provide novel therapeutic targets to reverse TRAIL resistance in NSCLC; (4) BID seems to be a major factor contributing to TRAIL-induced apoptosis with more influence than Bcl-2 and Mcl-1.

DECLARATIONS

Authors' contributions

Conceptualization: Peters GJ, Kruyt F, De Wilt L

Methodology: De Wilt L, Tabeian H

Validation: De Wilt L, Peters GJ, Kruyt F

Formal analysis: De Wilt L, Tabeian H

Investigation, resources, supervision, and project administration: Kruyt F, Peters GJ

Data curation: Jansen G, de Jong S, Peters GJ, Kruyt F

Writing - original draft preparation: De Wilt L, Sobocki BK

Writing - review and editing: Jansen G, de Jong S, Peters GJ, Kruyt F

Visualization: De Wilt L, Sobocki BK, Peters GJ

Funding acquisition: de Jong S, Kruyt F, Peters GJ

Availability of data and materials

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Conflicts of interest

Peters GJ is an Editorial Board Member of the journal *Cancer Drug Resistance*, while the other authors have declared that they have no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Supplementary Materials

[Supplementary Materials](#)

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KLF5 promotes tumor proliferation and oxaliplatin resistance via chromatin remodeling in KRAS-mutated colorectal cancer

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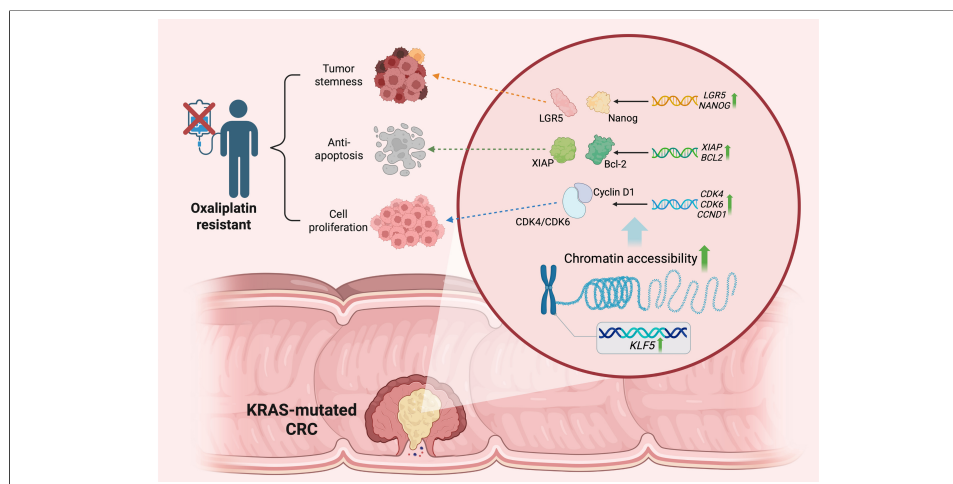
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Abstract

Aim: Patients with KRAS-mutated colorectal cancer (CRC) frequently exhibit resistance to conventional chemotherapy and epidermal growth factor receptor (EGFR)-targeted therapies. This study investigates the role of the transcription factor KLF5 in mediating proliferation and chemoresistance in KRAS-mutated CRC, aiming to identify novel therapeutic strategies to improve treatment outcomes.

Methods: We analyzed the association between KLF5 expression, KRAS mutation status, and patient prognosis using CRC tissue microarrays and public datasets. Proliferative capacity and oxaliplatin sensitivity were compared between KRAS-mutated and wild-type patient-derived organoids. RNA sequencing and CUT&Tag sequencing were employed to assess KLF5-mediated chromatin accessibility and downstream transcriptional regulation in KRAS-mutated CRC cells. *In vitro* and *in vivo* functional studies were conducted using three pairs of KRAS-mutated CRC cell lines (with KLF5 knockdown or overexpression) to evaluate KLF5's impact on proliferation, cell cycle progression, stemness, and oxaliplatin response.

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Results: KRAS-mutated CRC demonstrated enhanced proliferative capacity and oxaliplatin resistance, accompanied by KLF5 upregulation. In KRAS-mutated CRC cells, KLF5 promoted chromatin accessibility to initiate downstream transcription programs regulating cell cycle progression, platinum drug resistance, and apoptosis. Mechanistically, KLF5 drives oxaliplatin resistance by promoting proliferation through upregulation of the CDK4/6-Cyclin D1 axis, enhancing stemness via LGR5 and Nanog, and activating the XIAP/Bcl-2-dependent anti-apoptotic signaling pathway. *In vivo* experiments further confirmed that KLF5-overexpressing KRAS-mutated CRC tumors exhibited accelerated growth and reduced oxaliplatin sensitivity.

Conclusion: This study reveals that aberrantly elevated KLF5 promotes proliferation and chemoresistance in KRAS-mutated CRC. Targeting KLF5 represents a promising strategy to enhance chemotherapeutic response in this aggressive CRC subtype, offering a rationale for clinical translation.

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent malignancies worldwide, characterized by sequential genetic alterations in genes such as *APC*, *RAS*, and *TP53*^[1]. Approximately 40% of CRC patients harbor KRAS mutations^[2], leading to the constitutive activation of the KRAS protein and resistance to epidermal growth factor receptor (EGFR) inhibitors^[2]. Current standard therapies for KRAS-mutated CRC include surgical resection, chemotherapy, and targeted agents. Oxaliplatin-based regimens (e.g., FOLFOX, combining oxaliplatin with 5-fluorouracil and leucovorin) remain first-line chemotherapy options^[3]. However, chemoresistance is frequently observed in KRAS-mutated CRC, posing a significant clinical challenge.

KLF5, a zinc-finger transcription factor, plays pivotal roles in tumor cell proliferation, survival, apoptosis, and drug sensitivity^[4]. Recognized as an oncogenic driver, KLF5 is frequently overexpressed in CRC and promotes tumor progression^[5]. Our prior work demonstrated that the KLF5 inhibitor ML264 restores oxaliplatin sensitivity in chemoresistant CRC patient-derived organoids (PDOs) by suppressing the KLF5/Bcl-2/caspase3 anti-apoptotic axis^[6]. Similar oncogenic and therapy-resistant roles of KLF5 have been reported in esophageal squamous cell carcinoma^[7], prostate cancer^[8], ovarian cancer^[9], and breast cancer^[10]. Notably, KRAS-mutated CRC exhibits KLF5 upregulation, which correlates with aggressive tumor behavior^[11]. These findings suggest that KLF5 may serve as a critical mediator of malignant progression and chemoresistance in KRAS-mutated CRC, highlighting its therapeutic potential.

Gene expression networks in cancer cells are orchestrated by master regulatory transcription factors (MRTFs), which govern hallmark oncogenic traits such as sustained proliferation, evasion of growth suppression, and resistance to cell death^[12]. Emerging evidence positions KLF5 as an MRTF that regulates chromatin accessibility, epigenetic modifications, and transcriptional programs critical for tumor progression and microenvironment remodeling^[13,14]. Thus, KLF5 may drive CRC progression and chemoresistance by reshaping chromatin landscapes and activating oncogenic transcriptional networks, offering a mechanistic basis for targeting KLF5 to improve therapeutic outcomes in KRAS-mutated CRC.

METHODS

Cell lines and culture

This study utilized KRAS wild-type (WT) (RKO, HT29) and KRAS mutant (SW1116, SW480, SW620, HCT116, DLD1, LOVO) CRC cell lines, all obtained from the American Type Culture Collection (ATCC) with STR authentication. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine

serum (FBS) and maintained in a humidified incubator at 37 °C with 5% CO₂, following ATCC-recommended protocols^[4]. All cell-based experiments were regularly monitored for mycoplasma contamination.

Patients and specimens

This study was approved by the Biomedical Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, with written informed consent obtained from all enrolled CRC patients. Inclusion criteria required pathologically confirmed primary CRC without preoperative neoadjuvant therapy (radiotherapy or chemotherapy), and specimens were collected from laparoscopic surgical resections. Pathological TNM staging was performed according to the 2015 National Comprehensive Cancer Network (NCCN) guidelines.

Establishment and culture of patient-derived organoids from CRC patients

Tissue processing and patient-derived organoids (PDOs) generation were performed as previously described^[6]. Surgical specimens were washed with PBS containing penicillin-streptomycin, mechanically dissociated into small fragments, and digested with 0.5 mg/mL type IV collagenase in DMEM at 37 °C for 1 h. The digested suspension was sequentially filtered through 100 and 40 µm cell strainers, washed twice with PBS, and centrifuged to collect cells. Cell pellets were embedded in Matrigel and cultured with organoid medium containing 50 ng/mL EGF, 500 nM A83-01, 50 ng/mL Noggin, 3 µM SB202190, 10 nM prostaglandin E₂, and 1 × B27. PDOs were passaged using TrypLE™ Express to dissolve Matrigel.

Drug sensitivity assay

Drug screening was performed using stably passaged CRC PDOs. Approximately 50 PDOs embedded in Matrigel were seeded into 96-well plates and exposed to oxaliplatin (0–100 µM, covering clinically relevant plasma concentrations) or dimethyl sulfoxide (DMSO) vehicle control. Bright-field images were captured at baseline and on days 1, 3, 5, and 7 to monitor growth dynamics. Cell viability was quantified using CellTiter-Glo® Luminescent Assay. Dose-response curves were generated by nonlinear regression modeling, with results expressed as half-maximal inhibitory concentration (IC₅₀)^[15].

PDO growth evaluation

PDO growth was assessed as previously described^[6]. Bright-field images were captured on days 1, 3, 5, 7, 9, 11, and 13 post-Matrigel embedding. Organoid viability was assessed using CellTiter-Glo® Luminescent Assay, where ATP-dependent luminescence (relative light units, RLU) quantitatively reflected proliferative activity. RLU trajectories across time points were analyzed to generate growth curves and calculate population doubling time.

Histological staining analysis

Tissue specimens were fixed in 4% neutral buffered formalin, paraffin-embedded, and sectioned for H&E staining and immunohistochemistry (IHC). Antibodies used for IHC analysis included KLF5 and Ki-67. The TUNEL apoptosis detection kit was used according to the manufacturer's instructions. IHC results were semiquantitatively scored by two independent pathologists using the H-scoring system under blinded conditions. The CRC tissue microarray used in this study, containing 75 paired tumor and adjacent normal tissue samples, was constructed and validated as described in our prior publication^[4].

Bioinformatics analysis

The survival analysis was performed on the website <https://kmplot.com/analysis/>. The colon cancer database was used. RFS and OS were compared between KLF5-high and KLF5-low patients using the median expression level as the cut-off value.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Paired normal and tumor tissues from CRC patients were collected, flash-frozen in liquid nitrogen, mechanically dissected, and homogenized. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). cDNA was synthesized from total RNA using HiScript III RT SuperMix (Vazyme), and quantitative PCR was performed using SYBR Green (Vazyme) in accordance with the manufacturer's protocols. The qPCR primers were designed and supplied by Genewiz (China). The primer sequences were as follows: KLF5 Forward, 5'-ACACCAGACCGCAGCTCCA-3'; KLF5 Reverse, 5'-TCCATTGCTGCTGTCTGATTTGTAG-3'; GAPDH Forward, 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH Reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. GAPDH was used as the reference control gene. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Protein extraction and western blotting

Total proteins were extracted from CRC cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing protease/phosphatase inhibitors and quantified by bicinchoninic acid (BCA) assay. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After 1 h blocking with 5% non-fat milk, membranes were incubated with primary antibodies against KLF5, CDK1/2/4/6, Cyclin D1/D2/E1, p21/p16, LGR5, Nanog, SOX2, XIAP, BCL2, and loading controls (GAPDH) at 4 °C overnight. Following TBST washes, HRP-conjugated goat anti-rabbit/mouse secondary antibodies were applied for 2 h at room temperature. Signals were developed with ECL substrate, captured by a Tanon imaging system, and quantified using ImageJ software with normalization to loading controls.

siRNA transfection

KLF5-specific siRNA was transiently transfected into CRC cells using LipofectamineTM3000 following the manufacturer's protocol. Cells were harvested 48 h post-transfection, and knockdown efficiency was validated by qPCR assay. The most potent siRNA target sequence was selected to design the corresponding shRNA.

Lentiviral vector construction and validation

KLF5 overexpression (LV5-EF1a-GFP/Puro-KLF5) and knockdown (LV3-pGLV-h1-GFP/puro-sh-KLF5) lentiviral vectors, along with control vectors (LV5-EF1a-GFP/Puro-Vector and LV3-pGLV-h1-GFP/puro-sh-NC), were constructed by Shanghai GenePharma Co. Ltd. (China). Western blotting confirmed KLF5 protein modulation efficiency.

RNA-seq library preparation and bioinformatics analysis

Total RNA was isolated using TRIzol, treated with DNase I, and processed with Illumina Stranded mRNA Prep kit for library construction. Libraries passing Agilent 2100 Bioanalyzer QC were sequenced (150 bp paired-end) on Illumina NovaSeq 6000 (TIANGEN Biotech, China). Raw reads were quality-controlled via FastQC, trimmed using Trimmomatic, and aligned to GRCh38 with HISAT2. Gene expression quantification by featureCounts and differential analysis ($|\log_2 FC| \geq 1$, FDR ≤ 0.05) were performed using DESeq2 (v1.38.3). KEGG/GO enrichment of differentially expressed genes (DEGs) was analyzed via KOBAS (v3.0) and clusterProfiler (v4.6) with Bonferroni-corrected $P \leq 0.05$ ^[12].

CUT&Tag-Seq

The CUT&Tag assay was performed following established protocols^[12]. Briefly, 10^5 cells were gently washed twice with wash buffer and incubated with Concanavalin A-coated magnetic beads for 10 min at room temperature. After removing unbound supernatant, bead-bound cells were resuspended in dig wash buffer

and incubated overnight at 4 °C with a 1:50 dilution of primary antibody (normal mouse IgG) or control IgG on a rotating platform. Following primary antibody removal, cells were incubated with a 1:100 dilution of Rabbit Anti-Mouse IgG for 60 min. After 2-3 washes in dig wash buffer, cells were treated with a 1:100 dilution of pA-Tn5 adapter complex in dig-med buffer (0.01% digitonin, 20 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM spermidine, protease inhibitor cocktail) for 1 h. Subsequent washes in dig-med buffer were followed by tagmentation in 10 mM MgCl₂-containing buffer at 37 °C for 1 h. DNA was purified via phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. For library preparation, 21 µL of DNA was mixed with 2 µL of universal i5 and barcoded i7 primers, amplified using NEBNext HiFi 2× PCR Master Mix under the following conditions: 72 °C for 5 min (gap filling); 98 °C for 30 s; 14 cycles of 98 °C for 10 s and 63 °C for 30 s; final extension at 72 °C for 1 min. Libraries were purified with XP beads.

Cell viability assay

Cell proliferation was assessed using CCK-8 assay: CRC cells were seeded in 96-well plates (2×10^3 cells/well with 200 µL RPMI-1640 medium) and cultured for 24 h. Medium was replaced with fresh RPMI-1640 containing 10% CCK-8 reagent, followed by 2 h incubation at 37 °C in the dark. Absorbance was measured at 450 nm using a microplate reader. Relative cell viability was calculated against the 0 h baseline, with triplicate independent experiments.

Colony formation assay

Cells were seeded in 6-well plates at 1×10^3 cells/well and cultured in a 37 °C, 5% CO₂ incubator for 14 days. After gentle PBS washing, colonies were fixed with 4% paraformaldehyde (room temperature, 30 min), stained with 0.5% crystal violet for 2 h, rinsed with deionized water, and air-dried. Colonies containing ≥ 50 cells were imaged using an inverted microscope and quantified with ImageJ software, with triplicate independent experiments.

EdU cell proliferation assay

Cell proliferation was assessed using the EdU Cell Proliferation Kit. CRC cells were seeded in 6-well plates (5×10^4 cells/well) and cultured in RPMI-1640 medium for 24 h before treatment. Both treated and control cells were incubated with 50 µM EdU working solution in complete medium at 37 °C for 2 h, protected from light. After fixation with 4% paraformaldehyde and permeabilization with 0.5% Triton X-100, cells were reacted with Alexa Fluor 555-conjugated azide for 30 min in the dark. Nuclei were counterstained with Hoechst 33342. Three random fields per well were imaged using a fluorescence microscope. The ratio of EdU-positive cells was quantified using ImageJ software, with triplicate independent experiments.

Cell cycle analysis

Cells were fixed with 70% ice-cold ethanol, stained with 50 µg/mL propidium iodide (PI) and 100 µg/mL RNase A in the dark for 30 min. Analysis was performed on a BD FACSCanto II flow cytometer. DNA content histograms were analyzed to quantify the percentages of cells in the G₀/G₁ (diploid), S (DNA synthesis), and G₂/M (tetraploid) phases, with triplicate experiments.

Tumor spheroid formation assay

Cells were dissociated with 0.25% trypsin, resuspended in serum-free spheroid formation medium (DMEM/F12 supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, and 2% B-27® Supplement), and filtered through 40 µm cell strainers to obtain single-cell suspensions. Cells were seeded at 500 cells/well in ultra-low attachment 96-well plates and cultured at 37 °C, 5% CO₂ for 14 days with 50% medium replacement every 3 days. Spheroid morphology was observed using an inverted microscope, and spheroid formation rate was quantified using ImageJ software, with triplicate independent experiments.

Flow cytometry analysis

CRC cells were digested into single-cell suspensions using 0.25% trypsin/EDTA. The cells were washed with staining buffer (PBS containing 5% FBS) and resuspended in flow tubes at a concentration of 1×10^6 cells per 100 μ L. Cell staining was performed using fluorescently conjugated primary antibodies for 30 min at 4 °C in the dark. The antibodies used targeted CD44, CD133, and CD166. Samples were analyzed using a flow cytometer (BD Biosciences), and data were processed with FlowJo software (Tree Star).

Animal study

Four-week-old male BALB/c nude mice were obtained from Shanghai Laboratory Animal Center, CAS. Animal experiments were performed in strict compliance with the Guidelines for Animal Care and Use from Ruijin Hospital and Shanghai Resource Center of Laboratory Animals of the Chinese Academy of Sciences. All animal procedures in this study were performed at PHENOTEK Biotech (Shanghai) Co., Ltd, which is a commercially accredited animal facility commissioned by Ruijin Hospital. CRC cells (5×10^6) were subcutaneously injected into the right flank. When tumor volume reached 50 mm³, mice were randomized into control (saline) and oxaliplatin groups (5 mg/kg) for intraperitoneal injection twice weekly over 3 weeks. Tumor dimensions were measured every 5 days using digital calipers. Terminal euthanasia by CO₂ asphyxiation followed by cervical dislocation was performed, with excised tumors subjected to histopathological analysis ($n = 3$ /group).

Quantitative and statistical analysis

Data are presented as mean $\pm$ SD from three independent experiments. Flow cytometry data were analyzed using FlowJo V10.8.1. Statistical graphs were generated with GraphPad Prism 8.4.3. Two-group comparisons were performed using a two-tailed Student's *t*-test or the Mann-Whitney *U* test (nonparametric data). Multiple groups were analyzed using one-way ANOVA or two-way ANOVA as the experimental design required. Statistical significance was defined as $P < 0.05$.

RESULTS

KRAS-mutated CRC exhibits enhanced proliferative capacity and oxaliplatin resistance, accompanied by KLF5 upregulation

Patients with KRAS-mutated CRC exhibit a poor prognosis^[16]. However, limited studies have systematically evaluated the predictive value of KRAS mutations in determining responses to conventional chemotherapy regimens^[17]. We established PDOs from KRAS WT and mutant CRC tissues and evaluated their sensitivity to oxaliplatin. Drug response assays demonstrated that KRAS-mutant CRC PDOs exhibited significantly higher resistance to oxaliplatin compared to KRAS-WT counterparts, with elevated IC₅₀ values [Figure 1A-D]. Furthermore, KRAS-mutant PDOs displayed enhanced proliferative activity [Figure 1E]. Our prior study^[6] revealed that ML264, a KLF5-specific inhibitor, restored oxaliplatin sensitivity in CRC PDOs by suppressing the KLF5/Bcl-2/caspase3 anti-apoptotic signaling pathway. To assess the clinical relevance of KLF5 in CRC, we evaluated both mRNA and protein expression levels of KLF5 in paired tumor and adjacent normal tissues [Figure 1F-J]. While no significant difference in KLF5 expression was observed between T and N tissues overall [Figure 1F, I], KRAS-mutant CRC tissues exhibited markedly higher KLF5 levels compared to KRAS-WT tumors [Figure 1G and J]. To address the clinical relevance of KLF5 expression, we performed survival analyses across multiple public databases [Supplementary Figure 1]. The results showed that in aggregated databases, KLF5 expression did not exhibit a significant correlation with overall survival (OS) or relapse-free survival (RFS). However, in certain specific datasets, higher KLF5 expression was associated with worse OS (GSE16446) [Supplementary Figure 1B] or RFS (GSE25066 and GSE12276) [Supplementary Figure 1F and G]. Collectively, these findings demonstrate that KRAS-mutated CRC is characterized by hyperproliferation, oxaliplatin resistance, and specific KLF5 upregulation, suggesting KLF5 as a potential mediator of these oncogenic traits in KRAS-driven CRC.

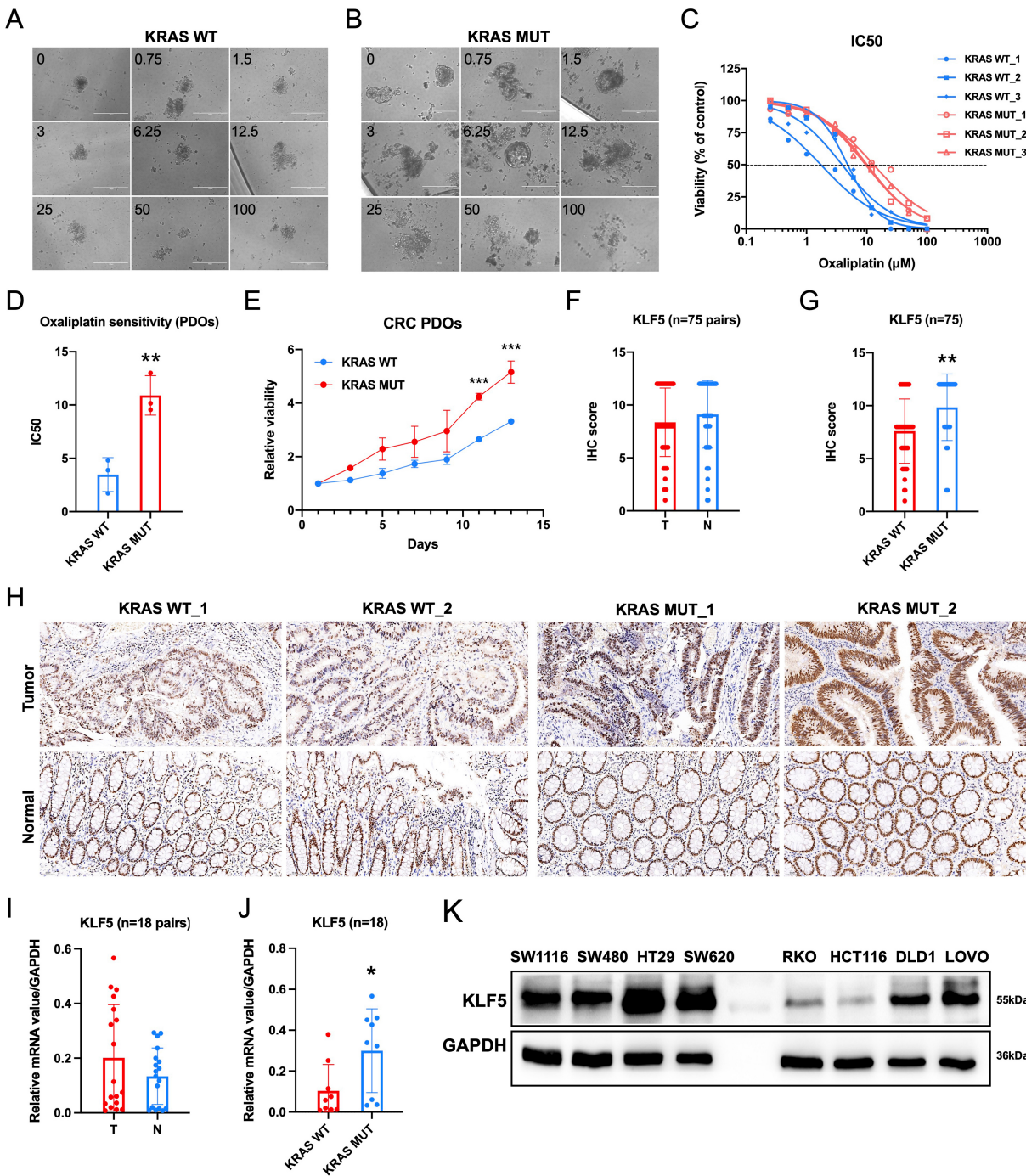


Figure 1. KRAS-mutant CRC exhibits elevated KLF5 expression, enhanced proliferation, and reduced sensitivity to oxaliplatin compared to KRAS wild-type CRC. (A) Oxaliplatin treatment in KRAS wild-type CRC; (B) Oxaliplatin treatment in KRAS-mutant CRC; (C and D) Dose-response curves demonstrating decreased oxaliplatin sensitivity in KRAS-mutant CRC, with significantly higher IC50 values compared to KRAS wild-type CRC; (E) Growth status of CRC PDOs, showing enhanced proliferative capacity in KRAS-mutant CRC PDOs; (F-H) Representative IHC staining images from a tissue microarray containing 75 paired CRC and adjacent N tissues. KLF5 expression shows no significant difference between T and adjacent N tissues, but is significantly upregulated in KRAS-mutant CRC; (I and J) qPCR analysis of KLF5 RNA expression levels in 18 paired CRC and adjacent N tissues. KLF5 mRNA expression shows no significant difference between T and adjacent N tissues, but is significantly upregulated in KRAS-mutant CRC; (K) KLF5 expression profiles in CRC cell lines. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. CRC: Colorectal cancer; KLF5: Krüppel-like factor 5; IC50: half maximal inhibitory concentration; PDOs: patient-derived organoids; IHC: immunohistochemistry; qPCR: quantitative polymerase chain reaction; RNA: ribonucleic acid; mRNA: messenger RNA; SD: standard deviation; T: tumor; N: normal.

KLF5 regulates cell cycle progression, platinum resistance, and apoptotic pathways in KRAS-mutated CRC

We first assessed KLF5 expression across eight CRC cell lines. In KRAS-WT cells, HT29 exhibited high KLF5 expression, while RKO showed low expression. Among KRAS-mutated cell lines, SW620, SW1116, HCT116, and LOVO displayed high KLF5 levels, whereas SW480 and DLD1 exhibited low expression [Figure 1K]. To investigate KLF5's functional role, we performed lentivirus-mediated shRNA knockdown in SW620 (KRAS-mutant, highest KLF5 expression), achieving significant KLF5 suppression [Figure 2A and B]. RNA sequencing (RNA-seq) analysis of control (SHNC) and KLF5-knockdown (SHKLF5) cells identified DEGs [fold change (FC) > 1.5, FDR < 0.01; Figure 2C-E]. Gene Ontology (GO) enrichment analysis of DEGs revealed top-ranked pathways including cell cycle regulation, positive regulation of transcription, DNA repair, and cyclin/CDK-positive transcription elongation factor complex activity [Figure 2F]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis further highlighted significant enrichment in Cell cycle, MAPK signaling, Platinum drug resistance, and Apoptosis pathways [Figure 2G]. These findings collectively suggest that KLF5 may be involved in modulating critical oncogenic processes in KRAS-mutated CRC, including cell cycle progression, platinum-based chemoresistance, and regulation of apoptotic pathways.

KLF5 enhances chromatin accessibility to activate downstream transcription in KRAS-mutated CRC

KLF5 is a transcription factor reported to be part of the core regulatory network in cancer, influencing chromatin accessibility, epigenetic modification, and gene expression patterns in tumor cells^[13]. To further investigate the molecular mechanisms by which KLF5 regulates cell cycle progression, platinum-based chemoresistance, and apoptosis in KRAS-mutated CRC, we performed CUT&Tag sequencing in SW620 control (SHNC) and KLF5-knockdown (SHKLF5) cells to genome-widely profile KLF5 binding sites and their effects on chromatin accessibility. After stringent quality control, high-quality clean data were obtained, with total reads of 46,253,136 (SHNC) and 42,161,811 (SHKLF5). Alignment using the BWA tool revealed unique mapping rates of 26.37% (SHNC) and 16.03% (SHKLF5). Peaks were called with FDR < 0.05, identifying 18,086 and 10,305 enriched peaks in SHNC and SHNC groups, respectively. Genomic distribution analysis of peaks suggested KLF5's potential functional targeting [Supplementary Figure 2A and B].

Using deeptools, we analyzed KLF5-associated chromatin signals across transcriptional start sites (TSSs), transcriptional end sites (TESs), and flanking 3 kb regions. Heatmaps demonstrated globally reduced signal intensity in the SHKLF5 group [Figures 3A and B, Supplementary Figure 2A and B], indicating KLF5's role in promoting chromatin accessibility. Peak localization analysis revealed predominant enrichment of KLF5 at promoter regions in CRC cells [Figures 3C, Supplementary Figure 2C and D]. GO and KEGG pathway analyses of peak-associated genes highlighted key pathways linked to KLF5 activity [Supplementary Figure 2E-H].

Integration of differential peaks (SHNC vs. SHKLF5) with RNA-seq DEGs identified 1,400 overlapping genes [Figure 3D]. KEGG enrichment of these genes implicated KLF5 in regulating WNT signaling, Platinum drug resistance, and Hippo signaling [Figure 3E]. GO analysis further linked these genes to cell cycle regulation, apoptosis, transcription coactivator activity, and protein binding [Figure 3F]. Integrative Genomics Viewer (IGV) genome browser snapshots displayed prominent CUT&Tag peaks at the promoter regions of key target genes. Compared to the SHKLF5 group (KLF5 knockdown), the NC group (normal control) exhibited stronger peak signals [Supplementary Figure 3A-D], indicating that KLF5 directly enhances chromatin accessibility at these target gene loci. Notably, aberrant activation of the WNT/ β -catenin pathway drives tumor progression and therapy resistance^[18], while Hippo signaling modulates tumor growth and drug response through proliferation and apoptosis control^[19]. These findings collectively demonstrate that KLF5

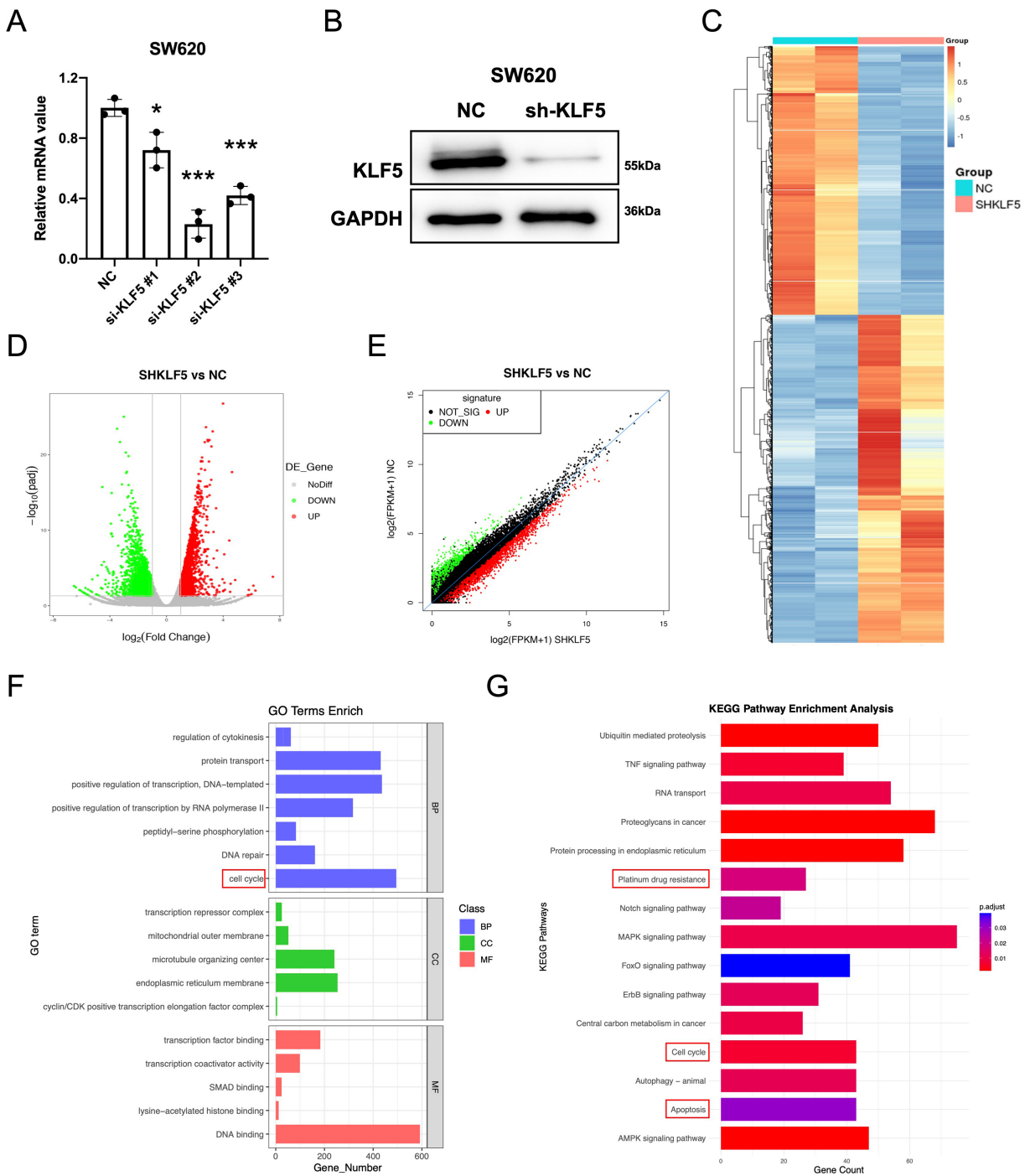


Figure 2. KLF5 regulates cell cycle, platinum drug resistance, and apoptosis-related pathways in KRAS-mutant CRC. (A) qPCR analysis of knockdown efficiency for different si-KLF5 sequences, with si-KLF5#2 showing the highest silencing efficacy; (B) Western blot validation of KLF5 knockdown efficiency; (C-E) RNA-seq analysis of differentially expressed genes (adjusted *P*-value < 0.05) in control (SHNC) and KLF5-knockdown (SHKLF5) KRAS-mutant CRC cells; (F) GO pathway analysis revealing top-ranked pathways altered upon KLF5 knockdown in SW620 cells; (G) KEGG pathway analysis highlighting the most significantly enriched pathways following KLF5 knockdown in SW620 cells. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; qPCR: quantitative polymerase chain reaction; siRNA: small interfering RNA; SHNC: short hairpin negative control; SHKLF5: short hairpin KLF5; RNA-seq: RNA sequencing; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SD: standard deviation.

facilitates chromatin opening to initiate downstream transcriptional programs that mediate proliferation and oxaliplatin resistance in KRAS-mutated CRC.

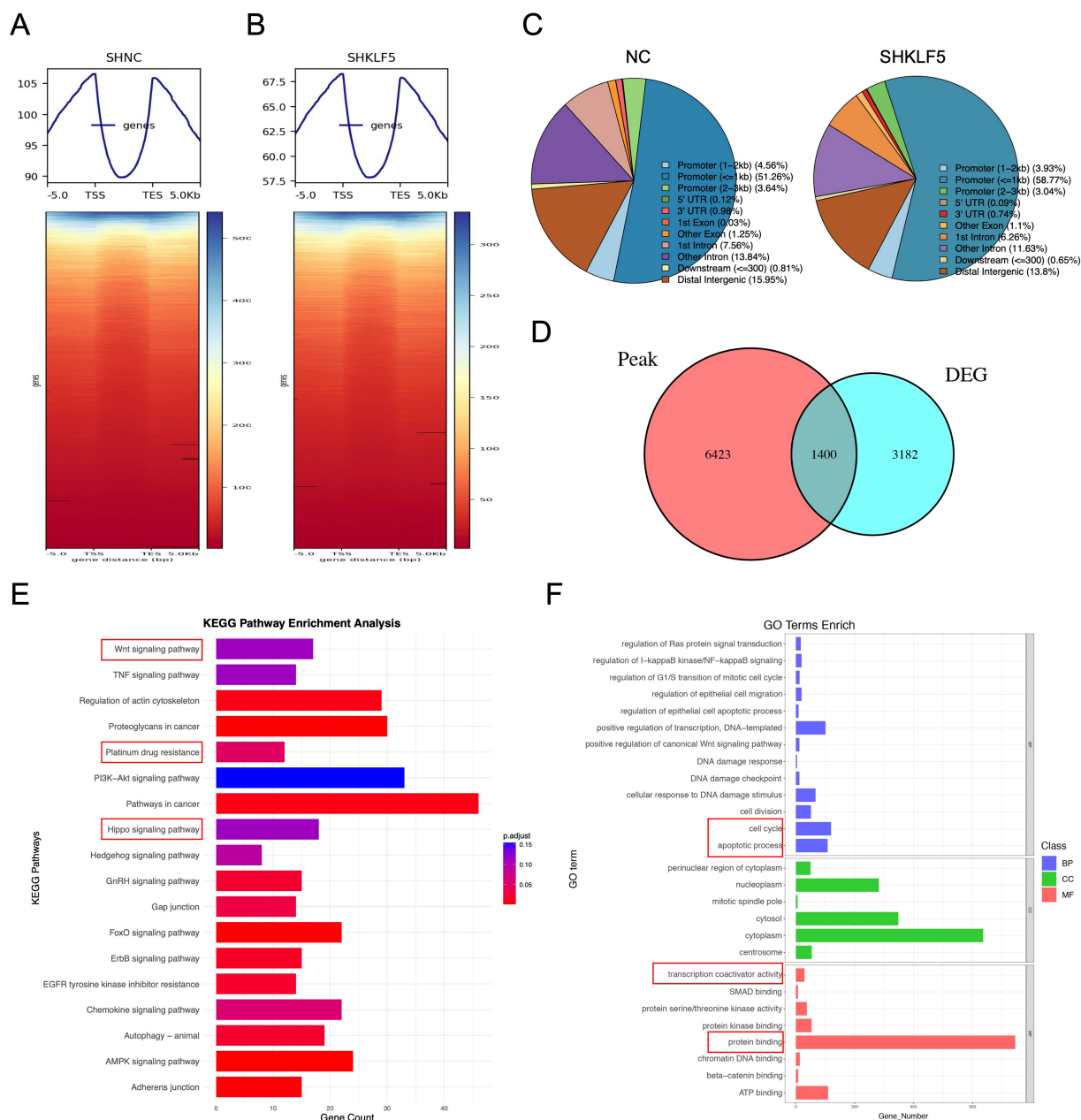


Figure 3. KLF5 promotes chromatin accessibility in KRAS-mutant CRC cells. (A and B) CUT&Tag sequencing analysis of control (SHNC) and KLF5-knockdown groups (SHKLF5). Signal distribution is calculated across TSSs, TESs, and flanking ± 3 kb regions, with corresponding heatmaps; (C) Pie chart summarizing the distribution of peaks across functional regions; (D) Venn diagram comparing differential peak-associated genes (SHKLF5 vs. SHNC) and RNA-seq-derived differentially expressed genes, identifying 1,400 overlapping genes; (E) GO pathway analysis of the overlapping genes; (F) KEGG pathway analysis of the overlapping genes. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; CUT&Tag: cleavage under targets and tagmentation; SHNC: short hairpin negative control; SHKLF5: short hairpin KLF5; TSS: transcription start site; TES: transcription end site; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

KLF5 promotes proliferation and cell cycle progression in KRAS-mutated CRC

To functionally validate KLF5's role in KRAS-mutated CRC, we transfected SW480 and HCT116 cells (KRAS-mutant, low KLF5 expression) with a KLF5 overexpression vector (oe-KLF5), achieving robust KLF5 upregulation as confirmed by Western blotting [Figure 4A]. Subsequently, we performed CCK-8 assays, EdU incorporation assays, and colony formation assays using stable CRC cell lines with KLF5 knockdown or overexpression, alongside their respective controls (SW480/oe-NC, SW480/oe-KLF5, SW620/sh-NC, SW620/sh-KLF5, HCT116/oe-NC, HCT116/oe-KLF5). CCK-8 proliferation assays demonstrated that KLF5

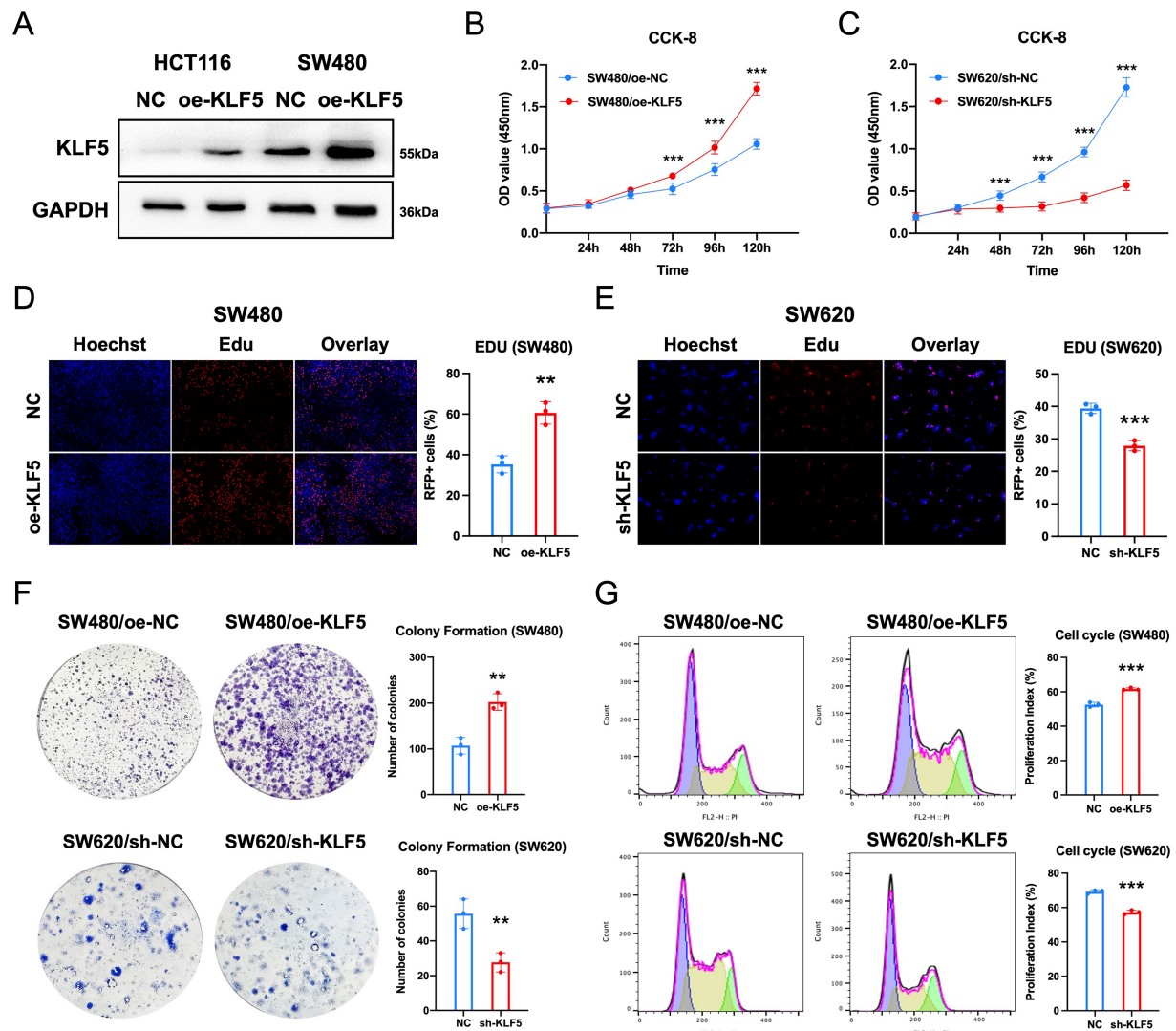


Figure 4. KLF5 promotes proliferation and cell cycle progression in KRAS-mutant CRC. (A) Western blot validation of KLF5 overexpression and knockdown efficiency; (B and C) CCK-8 assays assessing the effects of KLF5 on cellular proliferation; (D and E) EdU incorporation assays demonstrating KLF5-mediated regulation of proliferative activity; (F) Colony formation assay evaluating KLF5-dependent clonogenic capacity; (G) Cell cycle analysis revealing KLF5-driven modulation of cell cycle distribution. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; CCK-8: cell counting Kit-8; EdU: 5-ethynyl-2'-deoxyuridine; SD: standard deviation.

overexpression significantly enhanced cell viability, whereas KLF5 knockdown suppressed proliferation [Figure 4B and C, Supplementary Figure 4A]. Consistent results were observed in EdU incorporation assays [Figure 4D and E, Supplementary Figure 4B]. Colony formation assays further confirmed that KLF5 overexpression markedly increased clonogenic capacity, whereas KLF5 knockdown attenuated colony growth [Figure 4F]. Cell cycle analysis revealed that KLF5 overexpression accelerated cell cycle progression, whereas KLF5 knockdown induced G1-phase arrest [Figure 4G]. These data collectively demonstrate that KLF5 drives proliferation and cell cycle progression in KRAS-mutated CRC.

KLF5 drives proliferation and cell cycle progression in KRAS-mutated CRC via upregulation of the CDK4/6-Cyclin D1 axis

Analysis of RNA-seq data from SW620 control (SHNC) and KLF5-knockdown (SHKLF5) cells revealed that KLF5 upregulates cell cycle-related genes (CDK1, CDK6, CCND2, CCNB1) [Figure 5A]. Western blotting further demonstrated that KLF5 overexpression in SW480 cells increased protein levels of CDK4, CDK6,

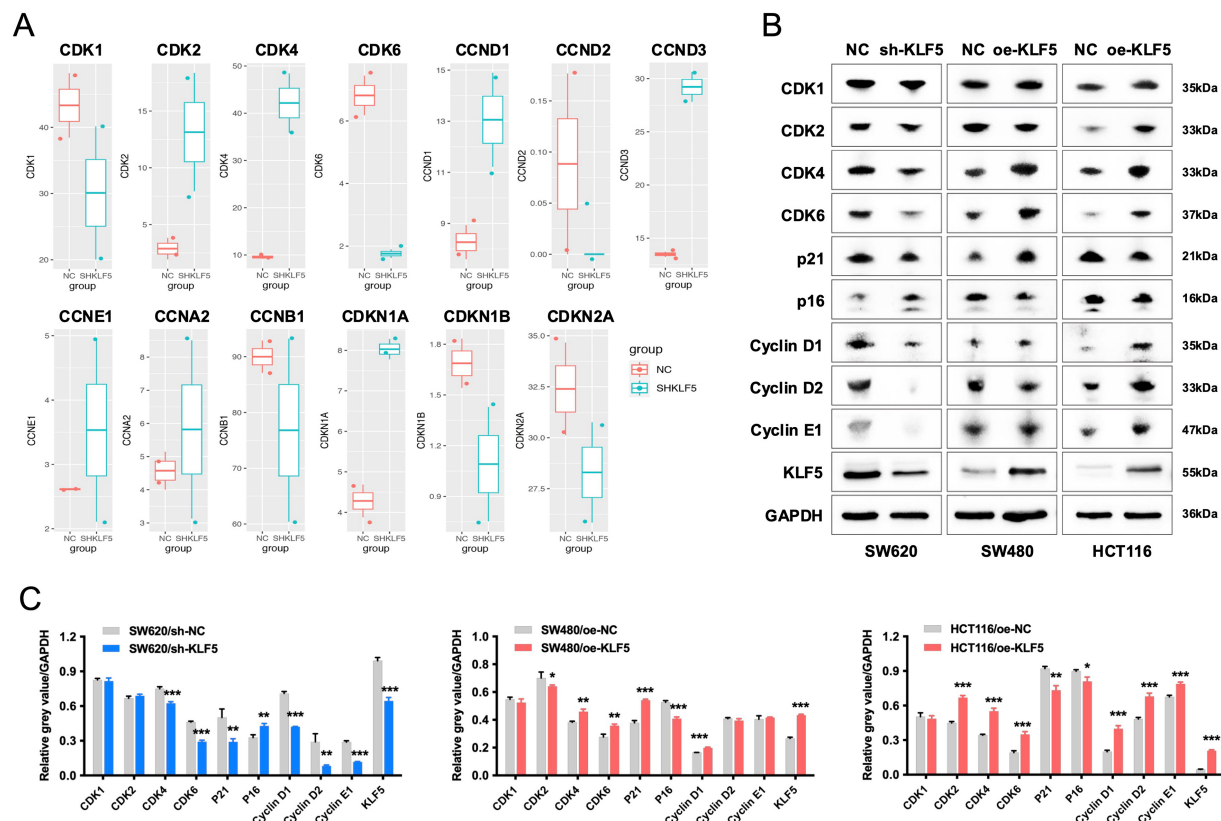


Figure 5. KLF5 drives proliferation and cell cycle progression in KRAS-mutated CRC via upregulation of the CDK4/6-Cyclin D1 axis. (A) RNA-seq analysis of differentially expressed cell cycle-related genes in KLF5-knockdown KRAS-mutant CRC cells; (B and C) Western blot analysis of KLF5, cell cycle regulators (CDK1, CDK2, CDK4, CDK6, Cyclin D1, Cyclin D2, Cyclin E1), and cell cycle inhibitors (p21, p16) in KRAS-mutant CRC cells, with GAPDH as a loading control. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; CDK: cyclin-dependent kinase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation.

Cyclin D1, and p21, whereas KLF5 knockdown in SW620 cells reduced the expression of these proteins. In TP53-wild-type, KRAS-mutant CRC cells (HCT116), KLF5 overexpression also upregulates CDK4, CDK6 and Cyclin D1, while downregulating the expression of cell cycle inhibitors p21 and p16 [Figure 5B and C]. CDK4 and CDK6, serine/threonine kinases, form complexes with Cyclin D1 to drive G1-to-S phase transition and promote proliferation^[20]. These findings establish that KLF5 enhances KRAS-mutated CRC proliferation and cell cycle progression by activating the CDK4/6-Cyclin D1 axis.

KLF5 promotes oxaliplatin resistance in KRAS-mutated CRC by enhancing tumor stemness and anti-apoptotic signaling

To investigate KLF5's role in chemoresistance, we assessed cancer stemness using spheroid formation assays. KLF5 overexpression in SW480 cells significantly enhanced spheroid-forming capacity, while KLF5 knockdown in SW620 cells markedly suppressed this phenotype [Figure 6A and B]. Oxaliplatin sensitivity assays demonstrated that KLF5 overexpression reduced drug responsiveness in SW480 cells, whereas KLF5 knockdown sensitized SW620 cells to oxaliplatin [Figure 6C and D]. We then quantified the expression of stemness surface markers CD133, CD166, and CD44 using flow cytometry, demonstrating that KLF5 overexpression increased the expression of these stemness markers in SW480 cells, while KLF5 knockdown suppressed their expression in SW620 cells [Figure 6E].

Analysis of RNA-seq data from SW620 control (SHNC) and KLF5-knockdown (SHKLF5) cells revealed that KLF5 upregulates the expression of XIAP and BCL2 while suppressing the expression of BCL2L1, BAX, and

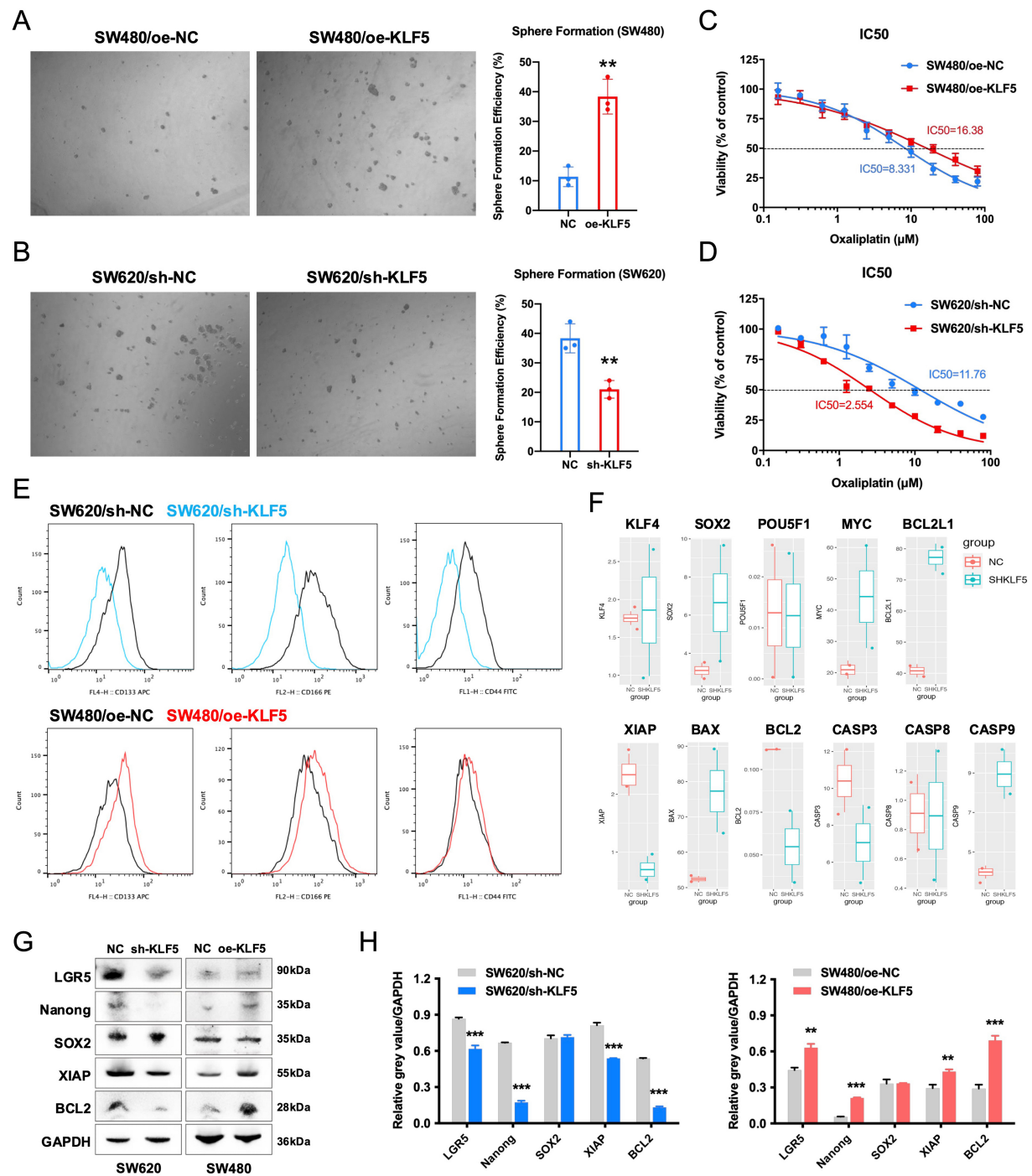


Figure 6. KLF5 enhances stemness and oxaliplatin resistance in KRAS-mutant CRC. (A and B) Sphere formation assays demonstrating KLF5-dependent regulation of cancer stem cell properties; (C and D) Dose-response curves of oxaliplatin treatment in KRAS-mutant CRC, highlighting KLF5-mediated drug resistance; (E) Flow cytometry analysis of stemness-related surface markers (CD133, CD166, and CD44) in CRC cells; (F) RNA-seq profiling of stemness- and apoptosis-associated genes altered by KLF5 knockdown in KRAS-mutant CRC cells; (G and H) Western blot analysis of stemness-related proteins (LGR5, Nanog, SOX2), and anti-apoptotic signaling molecules (XIAP and Bcl-2) in KRAS-mutant CRC cells, with GAPDH as a loading control. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; RNA-seq: RNA sequencing; LGR5: leucine-rich repeat-containing G-protein coupled receptor 5; SOX2: SRY-box transcription factor 2; XIAP: X-linked inhibitor of apoptosis protein; Bcl-2: B-cell lymphoma 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SD: standard deviation.

CASP9 [Figure 6F]. Mechanistically, BCL2 maintains mitochondrial outer membrane integrity by inhibiting pro-apoptotic BAX activation, thereby preventing cytochrome c release^[21]. XIAP directly binds caspases (e.g., CASP9, CASP3, CASP7) via its BIR domain to block apoptosis execution^[22]. These anti-apoptotic processes

have been demonstrated to mediate tumor resistance to oxaliplatin^[23-25]. Our prior work demonstrated that KLF5-mediated upregulation of Bcl-2 suppresses caspase3-dependent apoptosis, contributing to oxaliplatin resistance in CRC^[6]. Furthermore, Western blot analysis confirmed that KLF5 upregulates the expression of stemness-related genes (*LGR5* and *Nanog*) as well as anti-apoptotic signaling molecules (XIAP and Bcl-2) [Figure 6G and H]. These results demonstrate that KLF5 promotes oxaliplatin resistance in KRAS-mutated CRC by enhancing tumor stemness and activating anti-apoptotic signaling pathways.

KLF5 promotes tumor growth and oxaliplatin resistance in KRAS-mutated CRC *in vivo*

To validate KLF5's role *in vivo*, we established subcutaneous xenograft models by injecting KRAS-mutated CRC cell lines (SW480/oe-NC, SW480/oe-KLF5, SW620/sh-NC, SW620/sh-KLF5) into the flanks of nude mice. When tumors reached 50 mm³, mice received intraperitoneal oxaliplatin (5 mg/kg, twice weekly). KLF5 overexpression in SW480 tumors significantly accelerated tumor growth and conferred oxaliplatin resistance, whereas KLF5 knockdown in SW620 tumors suppressed tumor progression [Figure 7A-D]. Notably, the combination of KLF5 knockdown and oxaliplatin treatment synergistically suppressed tumor growth, achieving maximal therapeutic efficacy [Figure 7E and F]. These findings confirm that KLF5 drives tumor progression and chemoresistance in KRAS-mutated CRC *in vivo*.

As shown by Ki-67 IHC staining, KLF5 overexpression in SW480 tumors significantly promoted CRC cell proliferation, while KLF5 knockdown in SW620 tumors inhibited CRC cell proliferation. TUNEL staining revealed that KLF5 overexpression in SW480 tumors suppressed Oxaliplatin-induced apoptosis of CRC cells, whereas KLF5 knockdown in SW620 tumors enhanced Oxaliplatin-induced apoptosis of CRC cells [Figure 7G and H]. These findings demonstrate that KLF5 promotes proliferation of KRAS-mutated CRC *in vivo* and mediates Oxaliplatin resistance through anti-apoptotic mechanisms.

DISCUSSION

In the clinical management of KRAS-mutated CRC, frequent drug resistance is associated with aberrant sustained activation of signaling pathways such as EGFR^[26]. Emerging evidence highlights the critical role of transcriptional regulatory networks in driving progression and therapy resistance in EGFR-mutated tumors^[27]. Our study corroborates that KRAS-mutated CRC exhibits enhanced proliferative capacity and oxaliplatin resistance compared to KRAS wild-type counterparts. This underscores the potential of combining targeted therapies with conventional chemotherapy to improve therapeutic outcomes in KRAS-mutated CRC.

Our previous work demonstrated that ML264, a KLF5-specific inhibitor, restores oxaliplatin sensitivity in CRC PDOs by reactivating apoptotic responses via suppression of the KLF5/Bcl-2/caspase3 axis^[6]. Notably, multiple KLF5 inhibitors - including those targeting triple-negative breast cancer^[28], CRC^[29], osteosarcoma^[30], and pancreatic cancer^[31] - have shown potent antitumor activity, suggesting broad therapeutic potential. Building on these findings, we identified elevated KLF5 expression in KRAS-mutated vs. wild-type CRC. This study systematically evaluates KLF5's functional role in KRAS-mutated CRC, elucidates its mechanistic contributions to chemoresistance, and explores novel strategies to sensitize these tumors to chemotherapy.

Our study demonstrates that KLF5 promotes chromatin accessibility in KRAS-mutated CRC cells through RNA-seq and CUT&Tag sequencing analyses, initiating downstream transcriptional programs that regulate cell cycle progression, platinum drug resistance, and apoptotic pathways. Functional validation via *in vitro* and *in vivo* experiments further confirmed that KLF5 drives proliferation, cell cycle acceleration, stemness, and oxaliplatin resistance in KRAS-mutated CRC. Notably, we identified KLF5-mediated upregulation of the CDK4/6-Cyclin D1 axis as a critical mechanism for enhancing proliferation and cell cycle progression, while its suppression of apoptosis occurs through activation of anti-apoptotic proteins XIAP and BCL2. These

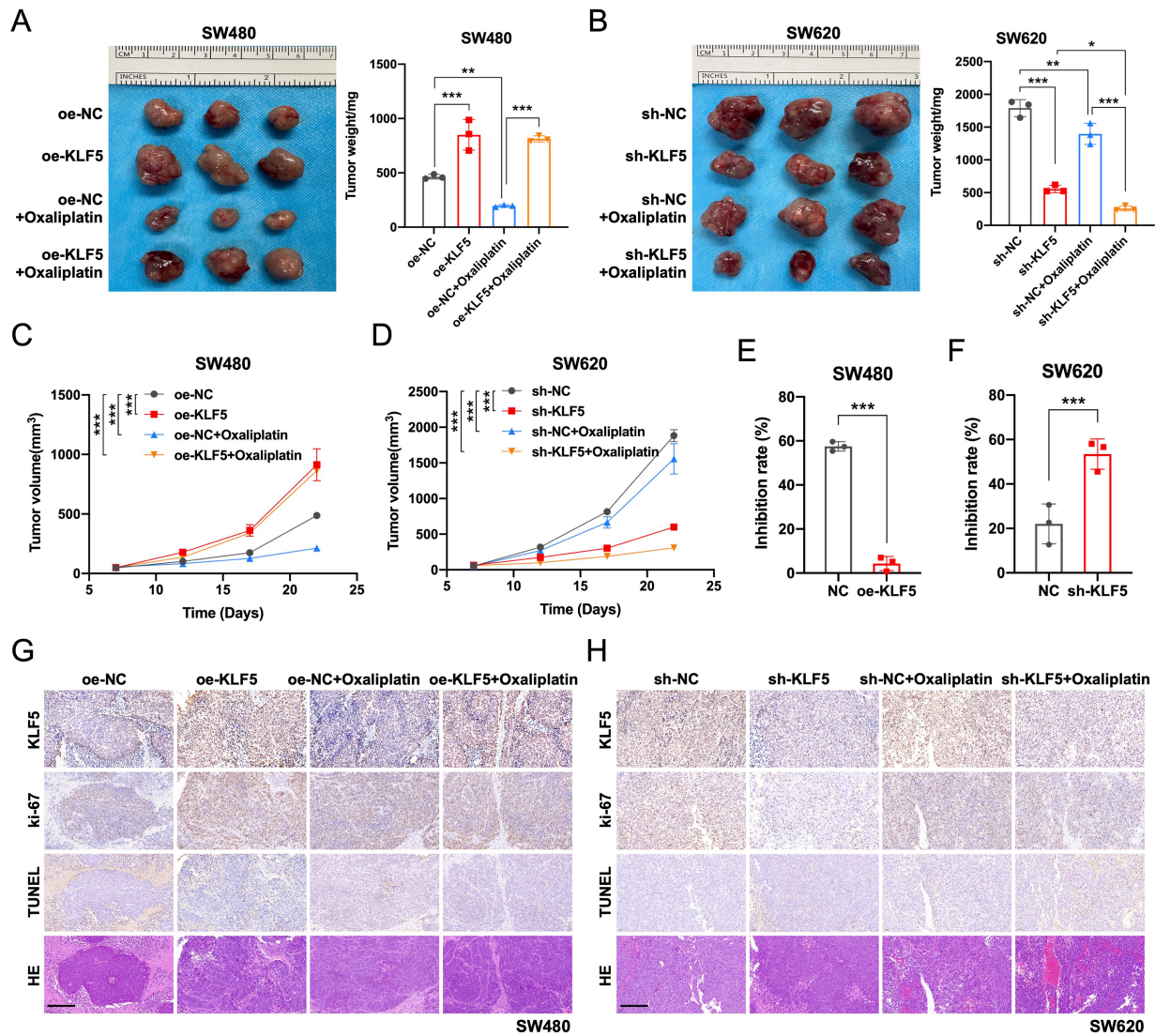


Figure 7. KLF5 drives tumor growth and suppresses oxaliplatin efficacy in KRAS-mutant CRC *in vivo*. (A and B) Xenograft tumor model established by injecting KRAS-mutant CRC cells into nude mice. Tumor volumes were calculated and plotted at the indicated time points (mean $\pm$ SEM); (C and D) KLF5 overexpression significantly promotes tumor growth and compromises oxaliplatin therapeutic effects in xenografts; (E and F) Assessment of Oxaliplatin tumor suppression efficiency in KRAS-mutated CRC tumors. KLF5 overexpression promoted Oxaliplatin resistance, while KLF5 knockdown synergistically enhanced tumor growth inhibition when combined with Oxaliplatin treatment; (G and H) IHC staining was performed to evaluate CRC tumor cell proliferation (Ki-67) and apoptosis levels (TUNEL). KLF5 overexpression significantly promoted CRC cell proliferation and suppressed oxaliplatin-induced apoptosis in CRC cells. Data represent the mean $\pm$ SD, Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. KLF5: Krüppel-like factor 5; CRC: colorectal cancer; SEM: standard error of the mean; IHC: immunohistochemistry; Ki-67: antigen Ki-67; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling; SD: standard deviation.

findings align with prior studies highlighting KLF5's regulatory roles in proliferation and apoptosis across cancers, yet they also underscore its context-dependent functional duality. For instance, KLF5 forms a complex with EHF/ELF3 in ovarian cancer to promote RAD51 transcription, enhancing homologous recombination repair and driving PARP inhibitor resistance^[9]. In breast cancer, a KLF5-FOXO1-XPO1 positive feedback loop regulates cell cycle and proliferation in basal-like subtypes^[10].

Conversely, KLF5 has been reported as a potential tumor suppressor in certain epithelial cancers. In androgen receptor-positive prostate cancer models, KLF5 knockdown paradoxically enhances proliferation^[32], while in p53-mutant esophageal squamous cell carcinoma, KLF5 transactivates CDKN1A

(p21) and NOTCH1, suppressing tumor growth^[33,34]. These contrasting roles suggest that KLF5's function is critically influenced by coexisting genetic alterations, particularly TP53 status. In our study, the KRAS-mutated CRC cell lines SW480 and SW620 harbor TP53 mutations. Although KLF5 upregulated p21 expression, no tumor-suppressive effects were observed, likely due to its dominant activation of the CDK4/6-Cyclin D1 axis, which overrides p21-mediated cell cycle arrest. This observation highlights the complexity of KLF5's regulatory network, where its oncogenic or tumor-suppressive outcomes depend on tissue-specific signaling contexts and genetic backgrounds. Our findings thus provide mechanistic insights into KLF5-driven chemoresistance in KRAS-mutated CRC and underscore the therapeutic potential of targeting KLF5 or its downstream effectors to overcome platinum-based therapy resistance.

Although KLF5 has been implicated in CRC risk^[35] and is reportedly overexpressed in CRC tissues, correlating with disease progression and poor prognosis^[36], our cohort revealed no significant difference in KLF5 expression between tumor and adjacent normal tissues. Notably, however, KLF5 was specifically upregulated in KRAS-mutated CRC. Recent studies have identified KLF5 as a critical transcriptional regulator in this molecular subtype^[11]. Furthermore, emerging evidence suggests that gene expression networks in cancer cells are governed by MRTFs, which orchestrate chromatin accessibility or cooperate with super-enhancers to sustain oncogenic programs such as proliferation, anti-apoptotic signaling, and drug resistance^[12]. Through CUT&Tag sequencing, we demonstrated that KLF5 promotes chromatin remodeling in KRAS-mutated CRC cells, initiating transcriptional activation of pathways critical for proliferation and oxaliplatin resistance. Collectively, this work establishes KLF5 as a pivotal driver of malignant phenotypes in KRAS-mutated CRC.

Nevertheless, our study has several limitations. For instance, high KLF5 expression was observed in certain KRAS wild-type CRC cells (e.g., HT29, which carries a BRAF mutation), suggesting that its strong expression might be driven by aberrant signaling in the Ras/Raf pathway rather than by KRAS status itself. However, the upstream regulatory mechanisms controlling KLF5 expression were not sufficiently explored in this work. Future studies should delineate the interplay between KRAS mutations and KLF5 regulation, as well as clarify KLF5's divergent roles in KRAS wild-type vs. mutant CRC. Additionally, validation of KLF5-targeted strategies in larger clinical cohorts and diverse preclinical models (e.g., patient-derived xenografts, organoids) is essential to confirm its therapeutic potential. Despite these limitations, our findings highlight the promise of KLF5 inhibition as a strategy to sensitize KRAS-mutated CRC to oxaliplatin, reinforcing KLF5's candidacy as a therapeutic target in this aggressive subset of CRC.

DECLARATIONS

Acknowledgments

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Authors' contributions

Conception and study design: Feng R, Shen X
Acquisition of data: Xu Z, Sun S, Gao H
Data analysis and interpretation: Sun S, Feng R
Statistical analysis: Gao H, Xu Z, Shen X
Manuscript preparation: Xu Z, Shen X

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its [Supplementary Materials](#). All data supporting our findings 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 was approved by the Biomedical Ethics Committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (No. 2020-315), with written informed consent obtained from all enrolled CRC patients. Animal experiments were performed in strict compliance with the Guidelines for Animal Care and Use from Ruijin Hospital and Shanghai Resource Center of Laboratory Animals of the Chinese Academy of Sciences. All animal procedures in this study were performed at PHENOTEK Biotech (Shanghai) Co., Ltd, which is a commercially accredited animal facility commissioned by Ruijin Hospital.

Consent for publication

Not applicable.

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Supplementary Materials

[Supplementary Materials](#)

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CF10/LV overcomes acquired resistance to 5-FU/LV in colorectal cancer cells through downregulation of the c-Myc/ABCB5 axis

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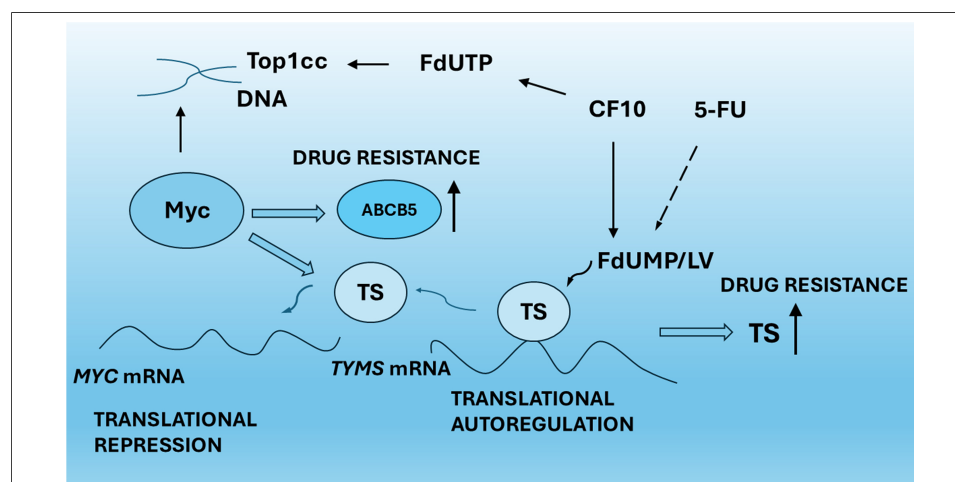
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Abstract

Aim: Acquired resistance to 5-fluorouracil/leucovorin (5-FU/LV) frequently develops during treatment of metastatic colorectal (mCRC), but the causes are incompletely understood. We aim to: (i) identify the causes of 5-FU/LV resistance under physiological folate; and (ii) determine if a polymeric fluoropyrimidine (FP) CF10 remains potent to CRC cells selected for 5-FU/LV resistance.

Methods: 5-FU/LV-resistant CRC cells were selected by repeated passaging with increasing 5-FU/LV concentrations, and resistance factors were calculated from dose-response studies. Basal and treatment-induced thymidylate synthase (TS), Myc, and ABCB5 were determined by RT-qPCR and Western blot. TS activity was determined using an *in situ* ³H-release assay. DNA topoisomerase 1 cleavage complexes (Top1cc) and DNA double-strand breaks (DSBs) were determined by immunofluorescence.

Results: Acquired resistance to 5-FU/LV with physiological folate was associated with a < 1.5-fold increase in basal TS levels; however, with either 5-FU/LV or CF10/LV treatment,



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TS levels were elevated ~5-fold by Western blot but only ~2-fold by RT-qPCR. CF10 remained very potent to CRC cells selected for 5-FU/LV resistance, and CF10 effectively induced TS ternary complex formation and inhibited TS catalytic activity in 5-FU/LV-resistant CRC cells. c-Myc was expressed at ~4-fold higher levels in 5-FU/LV-resistant CRC cells, but Myc was barely detectable with CF10/LV treatment. The Myc-target ABCB5, which is an established factor in resistance to 5-FU and other drugs, was substantially downregulated with CF10/LV but not 5-FU/LV treatment.

Conclusion: Acquired 5-FU/LV resistance was associated with FP-induced TS and elevated Myc and ABCB5. There is minimal cross-resistance to CF10 in 5-FU/LV-resistant CRC cells, consistent with its use in treating 5-FU/LV-resistant mCRC.

INTRODUCTION

The fluoropyrimidine (FP) drug 5-fluorouracil (5-FU) is central to the management of metastatic colorectal cancer (mCRC), with 5-FU-based regimens (FOLFOX, FOLFIRI) being administered to most mCRC patients^[1]. While the majority of mCRC patients initially respond to 5-FU-based chemotherapy, acquired resistance develops in nearly all patients^[2] [Figure 1]. Thus, while survival for mCRC patients enrolled in clinical trials now exceeds 30 months, during which many patients receive three or more lines of FP-based chemotherapy, long-term survival remains rare^[3]. An increased understanding of the causes of acquired 5-FU resistance thus remains an important objective in cancer research, as does the development of next-generation FP therapeutics to overcome the limitations of current 5-FU-based therapy^[4].

The primary molecular target of FP chemotherapy is thymidylate synthase (TS)^[5,6]. TS is required for *de novo* thymidylate biosynthesis, which promotes rapid cell proliferation in cancer cells. TS enzymatic activity requires the substrate dUMP and a reduced folate cofactor, N⁵,N¹⁰-methylene tetrahydrofolate (MeTHF), that serves as a carbon donor^[7]. The FP metabolite FdUMP forms a covalent complex with TS that inhibits enzymatic activity^[8]; however, 5-FU is inefficiently converted to FdUMP, with < 5% of the administered 5-FU converted to FdUMP^[9]. Irreversible TS inhibition also requires formation of a ternary complex that also includes a reduced folate cofactor^[10]. Humans have relatively low folate levels compared to rodents^[11], and folate levels are particularly low in cancer patients^[12,13]. For this reason, 5-FU is nearly always administered with LV (N⁵-formyl tetrahydrofolate) or newer reduced folate analogs that do not require metabolism^[14] for cancer treatment to promote TS inhibition despite 5-FU's inefficient conversion to FdUMP^[15].

Multiple studies have investigated the mechanistic basis for 5-FU resistance in CRC^[2,16-18]. A common theme among the majority of studies is that 5-FU resistance generally results from decreased TS inhibition that is due to either: (i) increased TS levels^[19]; (ii) decreased conversion of 5-FU to FdUMP^[20]; or (iii) increased breakdown of FdUMP or its cellular efflux [Figure 1]. To overcome resistance to 5-FU, we developed CF10^[21-24], a second-generation FP polymer that directly releases FdUMP. CF10 is much more potent than 5-FU against CRC cells (>300-fold on average in the NCI60 cell line screen)^[21], and this increased potency corresponds to stronger TS inhibition by CF10 at correspondingly lower doses relative to 5-FU. In previous studies, we have shown that FP polymers such as CF10 are highly effective in CRC cells selected for acquired resistance to 5-FU due to elevated TS expression^[25].

In addition to its enzymatic function, TS is an RNA-binding protein that autoregulates its own expression and the expression of other proteins, notably c-Myc and p53, through mRNA binding and translational repression^[26]. The function of TS as an RNA-binding protein is modulated by the formation of the FdUMP/MeTHF/TS ternary complex, which relieves autoregulation to enable increased TS expression under conditions of TS inhibition^[27]. The binding affinity of TS for its own mRNA and for *c-myc* is similar^[28].

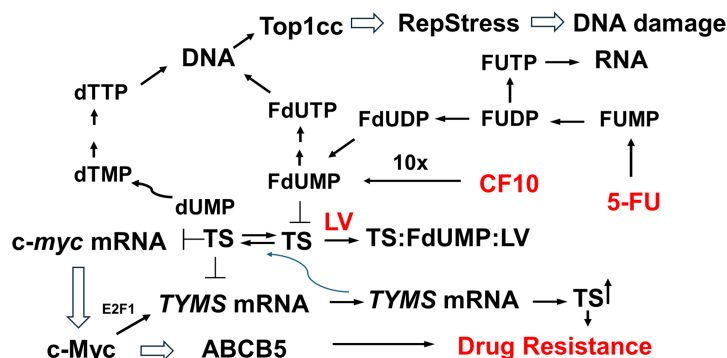


Figure 1. Resistance to 5-FU/LV converges on a lack of efficacy in inhibiting TS due to: (i) elevated TS expression; (ii) inefficient conversion to the TS inhibitory metabolite FdUMP; and/or (iii) activation of the c-Myc/ABCB5 axis. CF10 is efficiently converted to FdUMP, resulting in strong TS inhibition even when the enzyme is expressed at elevated levels. Figure preparation software is Microsoft PowerPoint (RRID: SCR_023631). 5-FU: 5-Fluorouracil; LV: leucovorin; TS: thymidylate synthase.

Hence, strong TS inhibition that reduces binding of TS mRNA could decrease c-Myc levels due to increased binding of c-myc mRNA by TS, inhibiting its translation. Decreased Myc levels in turn alter its translational program that is fundamental to determining the balance between proliferation and cell death^[29]. Among the Myc targets that have an established role in mediating resistance to 5-FU^[30] and other drugs is ABCB5^[31], which is involved in promoting a cancer stem cell phenotype^[32].

The purpose of this study is to characterize the role of TS elevation in CRC cells selected for acquired resistance to 5-FU/leucovorin (LV) under conditions of human-like folate levels and to determine if CF10 remains effective in these cells. Although multiple previous studies have investigated acquired 5-FU resistance^[2,33-35] and shown increased TS and *TYMS* levels, this is the first study to adapt cells to culture medium that includes human-like folate levels^[36] and to select for resistance to 5-FU/LV under conditions similar to those encountered clinically. We find that in multiple CRC cell lines, there is minimal constitutive change in TS at either the protein or mRNA level, but that 5-FU/LV treatment results in elevated TS levels that contribute to 5-FU/LV resistance. Given the interplay between TS and Myc regulation^[26], we also investigate perturbation in Myc^[37] and its direct transcriptional target ABCB5. We find that CF10 remains effective in CRC cells selected for 5-FU/LV resistance despite causing similar induction in TS, and this retained potency is associated with downregulation of c-Myc and ABCB5 at both the mRNA and protein levels.

METHODS

Cell lines and drugs

All parental colorectal cancer (CRC) cells [HCT116 (RRID:CVCL_0291), HCT15 (RRID:CVCL_0292), LS174T (RRID:CVCL_1384), and MC38 (RRID:CVCL_B288)] and 5FU-LV-resistant CRC cells (HCT116^R, HCT15^R, LS174T^R, MC-38^R) were cultured using folate-restricted (FR) media (folic acid-free RPMI 1640 supplemented with 40 nM of 5-methyltetrahydrofolate and 0.1 nM of sodium L-ascorbate)^[36], confirmed by short tandem repeat analysis, and repeatedly tested negative for Mycoplasma. The 5FU/LV-resistant CRC cells were generated by repeated passaging over 40 weeks in FR media supplemented with 5FU (0.1 to 10 μ M) and LV (10 μ M). Clinical-grade 5-FU (50 mg/mL) was purchased from the Baptist Hospital clinical pharmacy, and its concentrations were calculated based on the dilution of the established stock concentration. CF10 was obtained from ST Pharma, confirmed by high-resolution mass spectrometry, and dissolved in 0.9% sterile saline. Its concentrations were matched to deliver equivalent nucleoside content to an established dose of 5-FU based on UV absorbance at 260 nm.

Cell viability and resistance assay

CRC^P and CRC^R cells were seeded into white, flat-bottom 96-well plates and allowed to adhere and proliferate for 48 h until reaching about 25% confluency. To test for 5-FU/LV resistance, 2,500 cells per well, both the CRC Parental (CRC^P) and 5-FU/LV-resistant (CRC^R) cells, were seeded in 96-well plates and allowed to attach for 24 h. After 24 h, the cells were pre-treated for 2 h with LV before adding 5-FU and then incubated for 96 h at 37 °C in 5% CO₂. Cell viability was assessed using Aqueous-one (Promega) following the manufacturer's instructions. Resistance factors were calculated based on the ratio of IC50 values for CRC^P and CRC^R cells. All experiments were performed in triplicate.

Clonogenic assay

A modified clonogenic assay assessed the potency of CF10 and 5-FU in CRC^P and CRC^R cells. The CRC cells were plated in 24-well plates in FR media, and after 24 h, they were treated with the indicated concentration of CF10 or 5-FU for 72 h. The media were replaced after drug treatment, while the cells were allowed to grow for 168 h. Cell proliferation was evaluated using the Aqueous One (Promega) reagent, following the manufacturer's instructions, after the cell solution was transferred to 96-well plates. Apoptosis was evaluated using Caspase 3/7-Glo reagent (Promega) following the manufacturer's instructions.

Western blotting

Proteins were isolated, and their differential expressions were analyzed by Western blot, as described previously^[23]. Briefly, both CRC^P and CRC^R cells treated with LV, 5FU, 5FU + LV, CF10, or CF10 + LV were lysed using RIPA buffer (50 mM Tris HCl at pH 7.4, 150 mM NaCl, 1% Triton X-100 or NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 10 mM NaF freshly supplemented with protease and phosphatase inhibitors). The protein concentrations were quantified using a Bradford assay (Bio-Rad), and the samples were normalized for equal loading. All samples were then heated to 95 °C for 10 min in the presence of 6× Laemmli buffer (Boston Bio-Products; Milford, MA, USA). The proteins were resolved using SDS-PAGE, and their expression was analyzed following immunoblotting using specific antibodies. The following antibodies were used in this study: TS (Cell Signaling Technology Cat# 3766, RRID: AB_2210584), Myc (Cell Signaling Technology Cat# 18583, RRID:AB_2895543), ABCB5 (Thermo Fisher Scientific Cat# PA5-114801, RRID:AB_2899437), and β-actin (Santa Cruz Biotechnology Cat# sc-47778, RRID: AB_626632).

Immunoprecipitation assay

CRC^R treated with LV or 5FU + LV and non-treated cells were exposed to 100 nM control siRNA (Cell Signaling Technology Cat# 6568) and c-Myc siRNA (Cell Signaling Technology Cat# 6341) for 48 h as recommended by the vendor. The cells were lysed using RIPA buffer (50 mM Tris HCl at pH 7.4, 150 mM NaCl, 1% Triton X-100 or NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 10 mM NaF freshly supplemented with protease and phosphatase inhibitors). Immunoprecipitation [Thermo Scientific Pierce Co-Immunoprecipitation (Co-IP) Kit Cat# 26149] was performed using the cell lysates and ABCB5 (Thermo Fisher Scientific Cat# PA5-114801, RRID:AB_2899437) antibody. The enriched eluted lysates were resolved using SDS-PAGE and probed for ABCB5 protein expression (Thermo Fisher Scientific Cat# PA5-114801, RRID:AB_2899437).

Immunofluorescence Assay

Immunofluorescence detection of pH2AX (Cell Signaling Technology Cat# 2577, RRID:AB_2118010) was performed in drug-treated CRC^P and CRC^R cells that were fixed and permeabilized using 100% cold methanol and then processed as previously described^[38]. Top1cc were detected using a primary antibody specific to the cleavage complex (gift of Dr. S. Kaufmann) using procedures like those previously described^[38]. Top1cc foci were captured using Olympus Confocal Laser Scanning Microscope Fluoview

FV3000 (RRID:SCR_017015) and quantification of Top1cc foci was done via ImageJ by counting at least 100 cells that were declared positive if they had > 50 foci per nucleus.

RNA isolation and real-time PCR

Total RNA was isolated from CRC^P and CRC^R cells treated with LV, 5-FU, 5-FU + LV, CF10, or CF10 + LV using the RNeasy mini kit QIAGEN (RRID:SCR_008539). The Thermo Scientific NanoDrop One/OneC Microvolume UV Vis Spectrophotometer (RRID:SCR_023005) was used to determine the quality and quantity of RNA samples. cDNA synthesis was performed using the iScriptTM cDNA Synthesis Kit (Bio-Rad #:1708891). Real-time PCR was performed using the Bio-Rad CFX96 Real-Time PCR Detection System (RRID:SCR_018064) and the iTaq SYBR Green supermix (Bio-Rad #: 1725121). The primers for qRT-PCR were all purchased from OriGene Technologies Inc.

TS (human): Forward: 5'-GGTGTTTTGGAGGAGTTGCTGTG-3',
Reverse: 5'-GGAGAATCCCAGGCTGTCCAAA-3';
Myc (human): Forward: 5'-CCTGGTGCTCCATGAGGAGAC-3',
Reverse: 5'-CAGACTCTGACCTTTTGCCAGG-3';
ABCB5 (human): Forward: 5'-TTTGCCTATGCGGCAGGGTTTC-3',
Reverse: 5'-CAAACGAGCGTTTCTCCGATGG-3';
β-actin (human): Forward: 5'- CACCATTGGCAATGAGCGGTTC-3',
Reverse: 5'-AGGTCTTTGCGGATGTCCACGT-3'.

β-actin were used as an internal standard to normalize the relative expressions of the mRNAs. The RT-PCR reaction settings were Stage 1: Activation: 50 °C for 2 min; Stage 2: pre-soak:95 °C for 10 min; Stage 3: Denaturation: 95 °C for 15 s, Annealing: 60 °C for 1 min for 40 cycles; Stage 4: Melting curve: 95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s. The relative expressions were analyzed by the $2^{-\Delta\Delta C_t}$ method and experiments were performed in triplicate.

TS activity assay

A TS *in situ* activity assay was performed as previously described^[39] (PMID: 10554030) to quantitatively assess intracellular TS inhibition following drug treatment. Approximately 0.5×10^6 HCT-116 (RRID:CVCL_0291) and HCT116R cells were plated in each well of 6-well plates and allowed to attach for 24 h, then treated with either LV, 5-FU, 5-FU + LV, CF10, or CF10 + LV for 48 h. For the last 2 h, [³H]-2'-deoxyuridine (Moravek; 0.16 Ci/mmol; final concentration 2.5 μM) was added. After incubation, 150 μL of the culture media from each well was added to an ice-cold suspension containing 750 μL of charcoal with 0.5% T-70 dextran and 2.5% BSA, along with 150 μL of 35% trichloroacetic acid. The samples were centrifuged, and a portion of the supernatant was counted by liquid scintillation counting, and enzyme activities were expressed as a percentage relative to the drug-free control.

Statistical analysis

GraphPad Prism 10.2.0 GraphPad Prism (RRID:SCR_002798) was used for all statistical analyses. All experiments were conducted in triplicate, and data are presented as mean ± SEM. One-way ANOVA tests with recommended Tukey corrections were conducted, with $P < 0.05$ indicating significance.

RESULTS

Acquired resistance to 5-FU/LV occurs through treatment-induced TS elevation

In multiple previous studies, 5-FU-resistant CRC cells were generated through repeated passaging in media containing increasing concentrations of 5-FU^[2,33-35]. However, in the present studies, we repeatedly passaged human CRC cells (HCT116, LS174T, HCT15) in the presence of 5FU/LV, as 5-FU is nearly always used with

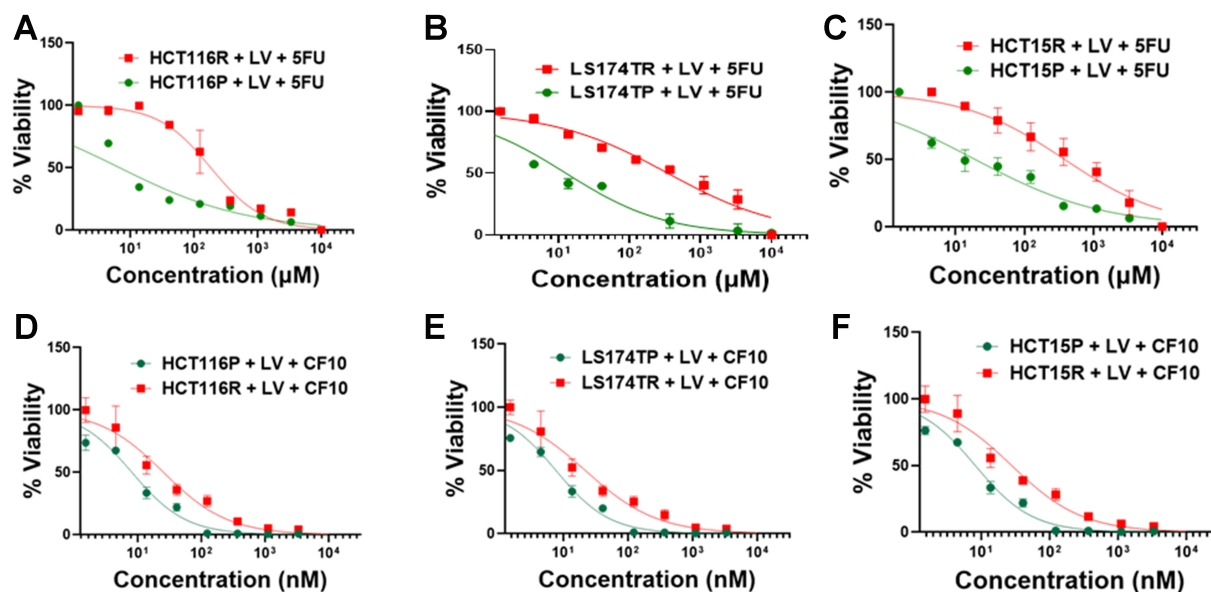


Figure 2. Dose response of parental (green) and 5-FU/LV-resistant (red) CRC cells to 5-FU/LV in (A) HCT116 ($P = 0.0022$), (B) LS174T ($P = 0.0028$), and (C) HCT15 ($P < 0.0001$) cells. Dose response to CF10/LV is shown in (D) HCT116 ($P = 0.0337$), (E) LS174T ($P = 0.0009$), and (F) HCT15 ($P = 0.0112$) parental (green) and 5-FU/LV-resistant (red) cells. IC₅₀ values and resistance factors for each CRC cell line are summarized in Table 1. Figure preparation software is GraphPad Prism (RRID: SCR_002798). 5-FU: 5-Fluorouracil; LV: leucovorin; CRC: colorectal cancer.

Table 1. Summary of IC₅₀ values for 5-FU/LV (μM) and CF10/LV (nM) and resistance factors

CRC cell line	(LV + 5FU) Treatment IC ₅₀ (μM)	Resistance factor	(LV + CF10) Treatment IC ₅₀ (nM)	Resistance factor
HCT116P	6.70		8.07	
HCT116R	183	27.3	25.3	3.14
LS174TP	12.8		7.73	
LS174TR	347	27.1	22.0	2.85
HCT15P	19.2		8.23	
HCT15R	383	19.9	28.0	3.40

5-FU: 5-Fluorouracil; LV: leucovorin; CRC: colorectal cancer.

LV in combination chemotherapy regimens for CRC. We also performed the selection by repeated passaging in media with restricted folate levels to better simulate human physiology. After selection by repeated passaging of parental cells (e.g. HCT116^P) over > 4 months in 5-FU/LV, we tested cells for the extent of resistance and found HCT116 5FU/LV-resistant (HCT116^R) cells were 27.3-fold less sensitive to 5-FU/LV relative to the corresponding parental cells [Figure 2A and Table 1]. Resistant LS174T and HCT15 cells showed similar resistance factors relative to the corresponding parental cells (27.1- and 19.9-fold) [Figure 2B and C, Table 1]. Importantly, all CRC cell lines selected for resistance to 5-FU/LV remained highly sensitive to CF10 and HCT116^R cells were only 3.14-fold less sensitive to CF10 than HCT116^P cells [Figure 2D], with other CRC cell lines showing similar moderate changes in sensitivity [Figure 2E and F, Table 1].

Since TS is an established target of 5-FU/LV, we performed Western blot and RT-qPCR to determine to what extent resistance in selected cells was associated with increased *TYMS* expression and increased TS protein levels. Quantification of Western blot images revealed that TS was upregulated ~2-fold in HCT116^R cells [Figure 3A and B] and similar increases were observed for LS174T [Supplementary Figures 1 and 2] and HCT15 cells [Supplementary Figures 3 and 4]. RT-qPCR revealed a modest increase in *TYMS* mRNA,

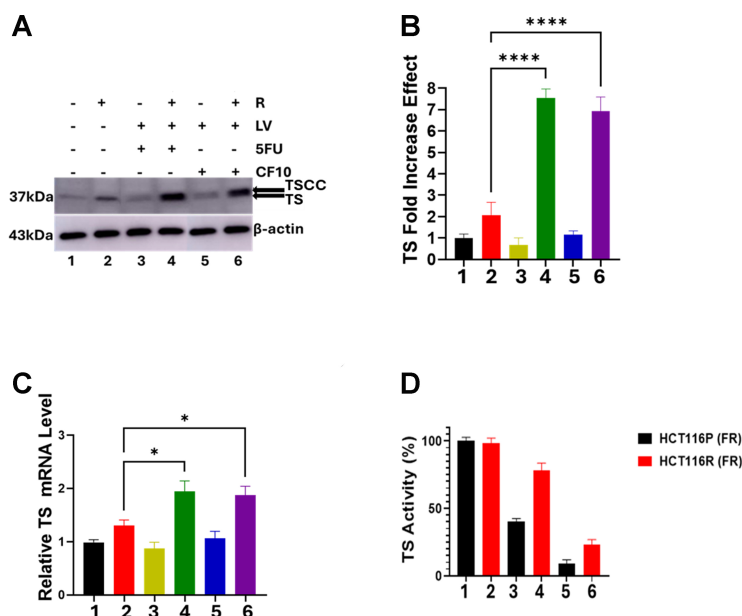


Figure 3. 5-FU/LV (10 mM, 1 mM) and CF10/LV (1 mM, 1 mM) treatment for 24 h increase TS at both the protein (A and B) and mRNA (C) levels in HCT116^R cells, but only CF10/LV promotes ternary complex formation (TSCC) and efficient inhibition of TS enzymatic activity (D). **P* < 0.03; *****P* < 0.0001. Figure preparation software is GraphPad Prism (RRID: SCR_002798). 5-FU: 5-Fluorouracil; LV: leucovorin; TS: thymidylate synthase; TSCC: TS classic complex.

~1.2-fold in HCT116^R cells [Figure 3C and Supplementary Figure 5] and in LS174T [Supplementary Figures 1 and 2], and HCT15 cells [Supplementary Figures 3 and 4]. We next investigated if TS levels were altered in response to treatment in FU/LV-R cells relative to parental cells. Treatment of HCT116^P cells resulted in no significant change in TS mRNA or protein levels under any of the treatment conditions tested. However, TS mRNA levels were increased in HCT116^R cells, with a similar ~2-fold elevation observed following both 5-FU/LV and CF10/LV treatments, regardless of the presence or absence of LV co-treatment [Figure 3C]. Similar trends were found for TS protein levels with 5-FU and CF10 treatment [Supplementary Figure 5], but the effects were larger at the protein level (~7-fold) than for *TYMS* mRNA (~2-fold) in HCT116^R cells [Figure 3A-C] and the other CRC cell lines selected for 5-FU/LV-resistance [Supplementary Figures 1-4]. Similar increases in TS were not detected with treatment in any of the parental CRC cell lines.

To determine if increased TS levels induced by 5-FU/LV or CF10/LV treatment in FU/LV-R CRC cells were a cause of resistance, we evaluated TS ternary complex formation based on decreased mobility on PAGE gels with Western blot detection [e.g., TS classic complex (TSCC)] [Figure 3A]. We also evaluated TS enzymatic activity using a TS *in-situ* enzyme activity assay (TSIA) based on ³H₂O formation from [5-³H]-dUMP [Figure 3D]. TS mobility in untreated and 5-FU/LV-treated cells was similar, indicating that 5-FU/LV treatment (10 mM, 1 mM; 24 h) did not effectively promote TS ternary complex (TSCC) formation in HCT116^R cells [Figure 3A]. In contrast, CF10/LV treatment (1 mM, 1 mM; 24 h) reduced TS mobility, consistent with TS ternary complex formation. CF10 was used at a 10-fold lower concentration than 5-FU to deliver equivalent FP levels. TSIA assays further demonstrated that CF10/LV effectively inhibited TS enzymatic activity in both HCT116^P and HCT116^R cells. Conversely, 5-FU/LV was less effective than CF10/LV at reducing TS enzymatic activity in HCT116^P cells and showed no significant inhibition in HCT116^R cells [Figure 3D]. Similar trends were observed with single-agent treatments in LS174T and HCT15 cells [Supplementary Figures 1-4] and in HCT116^R cells [Supplementary Figure 5]. Collectively, these studies showed that increased TS levels in CRC^R cells render 5-FU/LV ineffective at inducing TS ternary complex formation and inhibiting TS enzymatic activity, whereas CF10/LV remains effective in TS inhibition despite higher enzyme levels.

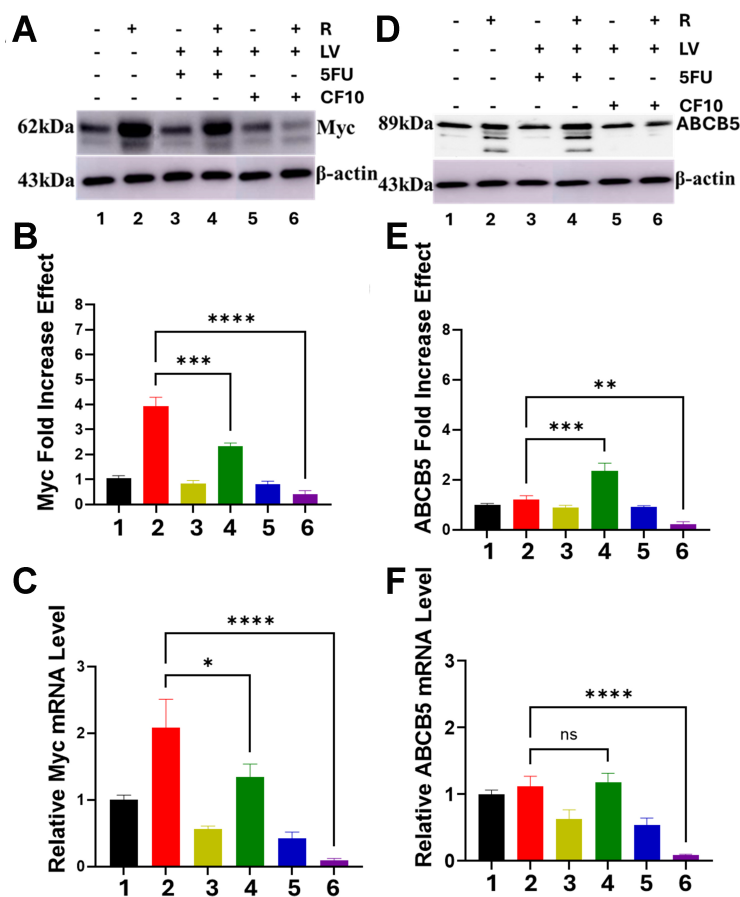


Figure 4. Myc is elevated in 5-FU/LV-resistant HCT116^R cells, but CF10/LV decreases levels of Myc and the Myc-target ABCB5 at both the protein and mRNA levels. (A) Western blot for Myc with quantification of the Western blot image by densitometry shown in (B) and quantification of mRNA levels by RT-qPCR shown in (C). (D) Western blot for ABCB5 with quantification of the Western blot image by densitometry shown in (E) and quantification of mRNA levels by RT-qPCR shown in (F). * $P < 0.03$, ** $P < 0.002$, *** $P < 0.0002$, **** $P < 0.0001$. Figure preparation software is GraphPad Prism (RRID: SCR_002798). 5-FU: 5-Fluorouracil; LV: leucovorin; RT-qPCR: reverse transcription quantitative polymerase chain reaction.

Myc is elevated in 5-FU/LV-resistant cells and is downregulated by CF10/LV

Myc is elevated in multiple cancers, including CRC, driving proliferation and upregulating a transcriptional program that contributes to drug resistance. Myc expression is translationally regulated by TS binding to its cognate mRNA^[28]; hence, treatments that affect TS levels could also alter Myc, affecting proliferation and drug resistance. The pattern of Myc expression in CRC^R cells parallels that for TS at baseline in the absence of treatment, with Myc levels ~4-fold higher at the protein level and ~2-fold higher at the mRNA level in HCT116^R cells compared to parental cells [Figure 4A-C, Supplementary Figure 6]. Similar trends were observed in LS174T and HCT15 cells [Supplementary Figures 7-10]. As for TS, treatment of parental cells with either 5-FU/LV or CF10/LV had minimal effect on Myc levels. However, in contrast to the increase in TS levels observed with treatment in HCT116^R cells, treatment decreased Myc at both the mRNA and protein levels in HCT116^R cells. 5-FU/LV treatment decreased Myc mRNA and protein relative to non-treated control cells; however, Myc levels were still elevated 1.5-fold (mRNA) and 2-fold (protein) in 5-FU/LV-treated HCT116^R cells relative to similarly treated HCT116^P cells [Figure 4A-C]. The effect of CF10/LV on Myc levels in HCT116^R cells was striking, with Myc barely detectable at either the mRNA or protein level in HCT116^R cells with CF10/LV treatment [Figure 4A-C]. Similar effects of CF10/LV on the reduction of Myc levels were detected in LS174T^R and HCT15^R cells [Supplementary Figures 7-10].

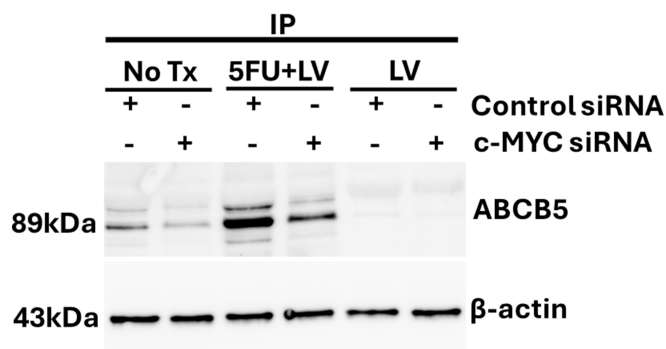


Figure 5. Immunoprecipitation of HCT116^R cell lysates reveals decreased ABCB5 following siRNA knockdown of Myc, consistent with increased ABCB5 in HCT116^R cells with 5-FU/LV treatment resulting from Myc-mediated transcription. Figure presentation software is the Bio-Rad ChemiDoc MP Imaging System (RRID: SCR_019037). 5-FU: 5-Fluorouracil; LV: leucovorin.

The effects of elevated Myc in mediating resistance to 5-FU and other drugs have implicated ABCB5 as an important direct target of Myc that mediates drug resistance^[30]. To determine if ABCB5 levels were altered in CRC cells selected for 5-FU/LV acquired resistance, we evaluated ABCB5 levels both by Western blot [Figure 4D and E, Supplementary Figure 6] and RT-qPCR [Figure 4F and Supplementary Figure 6]. The principal difference in Western blots for ABCB5 in 5-FU/LV-resistant cells relative to parental cells was the appearance of three additional bands with slightly increased mobility relative to the major band detected at ~89 kDa. ABCB5 undergoes alternative splicing to produce multiple protein products, and the new bands detected in 5-FU/LV-resistant CRC cells are likely due to alternatively spliced products (proteomics analysis is in progress). Treatment with 5-FU/LV resulted in slight intensification of one band [Figure 4D and E]. In contrast, none of the additional bands attributed to ABCB5 alternative splicing were detected in HCT116^R cells treated with CF10/LV. Furthermore, CF10/LV treatment reduced ABCB5 mRNA levels to barely detectable levels, similar to what was observed for Myc mRNA in HCT116^R cells following CF10/LV treatment.

Myc siRNA knockdown decreases ABCB5 in CRC^R cells

To demonstrate that ABCB5 is a Myc target in CRC^R cells, we performed siRNA-mediated knockdown of Myc followed by immunoprecipitation with anti-ABCB5. The proteins in the cell lysates were then separated by SDS-PAGE and probed to detect ABCB5 expression. As shown in Figure 5, treatment of HCT116^R cells with 5-FU/LV increased ABCB5 levels as expected; however, treatment with c-Myc siRNA reduced ABCB5 to basal levels comparable to untreated cells. These findings suggest that the increase in ABCB5 expression observed after 5-FU/LV treatment in HCT116^R cells is mediated by enhanced Myc-dependent transcription.

CF10/LV induces Top1cc and DNA double-strand breaks in 5-FU/LV-resistant CRC cells

CF10 was shown to induce DNA topoisomerase 1 cleavage complex (Top1cc) formation in CRC cells^[21], and it displayed mechanistic similarities to Top1cc poisons such as camptothecin in a COMPARE analysis of data from the NCI60 cell line screen^[40,41]. To determine if Top1cc formation and induction of DNA double-strand breaks (DSBs) also occur in CRC cells selected for 5-FU/LV-resistance upon treatment with CF10/LV, we performed immunofluorescence studies using antibodies specific for Top1cc^[38] and γH2AX, a biomarker of DNA DSBs [Figure 6, Supplementary Figures 11–16]. Top1cc were evident in nearly all HCT116^R cells treated with CF10/LV. Similarly, γH2AX immunostaining was evident in nearly all CF10/LV-treated HCT116-R cells, but not in those treated with 5-FU/LV. Our results are consistent with CF10/LV cytotoxicity being mediated through dual targeting of TS/Top1 in CRC cells selected for acquired 5-FU/LV resistance, whereas 5-FU/LV alone is not effective in causing DNA DSBs in these cells.

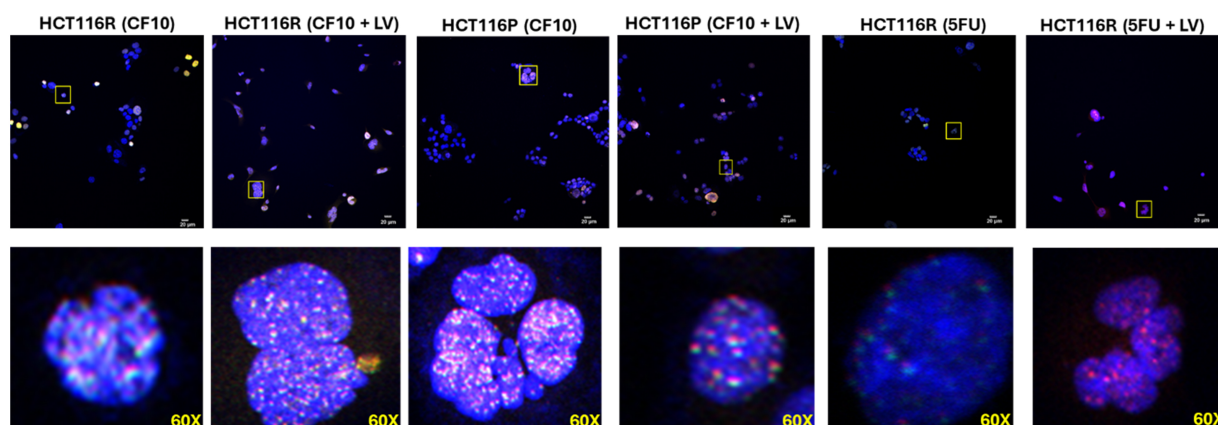


Figure 6. CF10/LV is effective at inducing Top1cc and γ H2AX, a biomarker of DNA DSBs in both HCT116^R and HCT116^P cells, consistent with TS/Top1 dual targeting by CF10 in 5-FU/LV-resistant cells while 5-FU is relatively ineffective at causing DNA DSBs. Shown are merged images for DAPI (blue), γ H2AX (green), and Top1cc (red) for the indicated cell lines and treatment (5-FU 10 mM, CF10 1 mM, LV 1 mM) for 24 h. Complete images are included in [Supplementary Figures 11–16](#). Figure presentation software is Olympus Confocal Laser Scanning Microscope Fluoview FV3000 (RRID: SCR_017015). LV: Leucovorin; DSBs: double-strand breaks; TS: thymidylate synthase; 5-FU: 5-fluorouracil.

DISCUSSION

This study characterizes three human CRC cell lines (HCT116, LS174T, HCT15) selected for acquired resistance to 5-FU/LV [Figure 1] in culture media that simulate human-like folate levels (FR media). All three CRC cell lines developed similar levels of 5-FU resistance (19.9–27.3-fold) [Figure 2 and Table 1], and these resistance levels were similar to those of CRC cell lines selected for resistance to 5-FU in standard media^[2]. Because of its established role as a primary target of FP chemotherapy and mediator of 5-FU resistance, we evaluated TS expression at the protein and mRNA levels at baseline and in response to treatment with either 5-FU or a next-generation FP polymer CF10, with and without LV co-treatment. In contrast to previous studies, in which CRC cells selected for acquired resistance to 5-FU displayed elevated TS levels at baseline^[34], the CRC^R cells selected for acquired resistance to 5-FU/LV showed no significant increase in TS at either the mRNA or protein level in the absence of treatment. However, treatment with 5-FU/LV and, to a lesser extent, CF10/LV increased TS levels, and the effects were larger at the protein level than at the mRNA level [Figure 3, [Supplementary Figures 1–5](#)]. The mechanism by which 5-FU/LV treatment stimulates increased TS in CRC^R cells is under investigation in our laboratory. One possible explanation is treatment-induced stabilization of TS protein, preventing its degradation. TS can be stabilized against proteasome-mediated degradation through O-GlcNAc conjugation^[42], and it is possible that 5-FU/LV treatment enhances TS O-GlcNAc conjugation, thereby increasing protein stability.

In addition to its role in *de novo* thymidylate biosynthesis, TS is an RNA-binding protein that regulates its own expression and the expression of c-Myc and p53. Myc and p53 are key proteins that regulate cell proliferation and programmed cell death, and alterations in TS levels and in TS ternary complex formation could mediate drug resistance, in part by modulating c-Myc and p53 levels. In fact, c-Myc is significantly elevated in CRC^R cells selected for acquired resistance to 5-FU/LV relative to parental cells [Figure 4]. Although Myc levels were also altered in response to treatment, in contrast to the increased TS levels observed in response to 5-FU/LV treatment in CRC^R cells, both 5-FU/LV and CF10/LV significantly decreased Myc protein levels. Additionally, 5-FU/LV and CF10/LV treatments significantly decreased Myc mRNA levels, with Myc mRNA levels becoming barely detectable after CF10/LV treatment as measured by RT-qPCR. A possible explanation for these observations is that CF10/LV efficiently promotes the TS ternary complex, which relieves TS autoregulation^[27] and increases TS binding to Myc mRNA, thereby decreasing its translation. However, this process does not fully explain the observed decrease in Myc mRNA levels,

particularly with CF10/LV treatment [Figure 4A-C, Supplementary Figures 7-10], indicating that additional processes are involved. Furthermore, while 5-FU/LV treatment reduces Myc levels in CRC^R cells, it simultaneously enhances Myc's transcriptional activity with respect to ABCB5 expression [Figure 5]. Thus, both the levels and transcriptional activity of Myc are altered by 5-FU/LV treatment in CRC^R cells.

CF10 causes Top1cc and DNA DSB formation [Figure 6] and induces cell-cycle arrest, which could contribute to decreased c-Myc expression. In contrast, 5-FU/LV is less effective at promoting TS ternary complex formation [Figure 3] and induces lower levels of DNA DSBs [Figure 6], consistent with reduced cell-cycle arrest and correspondingly decreased Myc expression. While Myc regulates multiple targets involved in drug resistance, its direct transcriptional control of ABCB5 expression is known to modulate resistance to 5-FU in CRC cells^[30]. In our study, we detected multiple higher-mobility bands for ABCB5 in Western blots of CRC^R cells [Figure 4D and E, Supplementary Figures 7-10], which may reflect alternative splicing events previously reported for ABCB5^[31]. However, to our knowledge, these splice variants have not been implicated in acquired resistance to 5-FU/LV. Proteomic analyses are ongoing to identify the altered forms of ABCB5 in CRC^R cells. Interestingly, CF10/LV treatment decreased ABCB5 expression to barely detectable levels by RT-qPCR, and the putative alternatively spliced forms were undetectable following CF10/LV treatment. Collectively, these results suggest that CF10/LV downregulates the Myc/ABCB5 axis in CRC^R cells, thereby promoting strong cytotoxicity and overcoming acquired resistance to 5-FU/LV.

Acquired resistance to 5-FU/LV is a complex phenomenon, and our study necessarily focused on a limited number factors, particularly the induction of TS protein levels following 5-FU/LV treatment [Figure 3], and the increased basal levels of Myc in 5-FU/LV-resistant cells, which are modulated by FP treatment [Figure 4]. Myc has established roles in 5-FU resistance mediated through ABCB5, which we showed is differently affected by 5-FU/LV and CF10/LV treatments. Further investigation is needed to clarify how CF10/LV differentially regulates Myc levels relative to 5-FU/LV. In addition, Myc interacts with topoisomerase 1, and excessive Myc-topoisomerase activity can induce DNA damage and promote Myc degradation^[43]. Top1 is also a target of CF10/LV [Figure 6], and future studies will investigate whether the decreased Myc levels observed with CF10/LV treatment involve this Myc-topoisomerase interaction. Another limitation of our study is that it was limited to cell models of 5-FU/LV resistance. Future research will extend these findings to *in vivo* models to validate the results and assess their potential impact on the standard of care for mCRC treatment.

The mortality associated with CRC results almost exclusively from metastatic disease. 5-FU/LV is widely used in regimens such as FOLFOX and FOLFIRI for the treatment of mCRC^[1], and the majority of patients initially respond to first-line chemotherapy. However, acquired resistance to 5-FU/LV almost invariably develops and long-term survival remains uncommon. In previous studies, we demonstrated that CF10 was very well tolerated *in vivo*, inducing less weight loss and causing fewer GI-tract and hematological toxicities compared to equivalent doses of 5-FU. Moreover, CF10 displayed superior antitumor activity relative to 5-FU in mouse models of primary CRC^[21] and in mouse and rat models of liver-metastatic CRC^[23]. In these studies, we also evaluated whether CF10 remained effective in CRC cells selected for acquired resistance to 5-FU/LV. Remarkably, the resistance factors for CF10 were only 2.85- to 3.40-fold higher, approximately an order of magnitude lower than those for 5-FU in these cell lines [Table 1]. CF10 maintained its potency despite inducing similar increases in TS levels as 5-FU in CRC^R cells [Figure 3A and B, Supplementary Figures 7-10]. This retained efficacy was correlated with: (i) increased formation of the TS ternary complex in CRC^R cells [Figure 3A] and (ii) decreased TS enzymatic activity [Figure 3D]. The strong TS inhibition achieved by CF10/LV in CRC^R cells corresponded to the formation of Top1cc, as previously observed in CRC^[21] and pancreatic cancer cells^[22]. This effect results from FdU misincorporation into DNA, which inhibits the religation step of Top1 catalysis^[40]. Additionally, CF10/LV was significantly more potent than 5-FU/LV at inducing γ H2AX foci [Figure 5], a biomarker of DNA DSBs, consistent with dual targeting of

TS/Top1, causing replication stress and subsequent replication fork stalling and collapse. In summary, our results provide new insights into the mechanisms underlying acquired resistance to 5-FU/LV in CRC cells and indicate that CF10/LV is an effective alternative. Ongoing studies aim to test the efficacy of CF10/LV in *in vivo* models of acquired 5-FU/LV resistance and to advance CF10 into clinical trials. The activity demonstrated by CF10/LV in CRC cells selected for acquired resistance to 5-FU/LV supports its potential for clinical development, including use in patients previously treated with 5-FU/LV-based chemotherapy such as FOLFOX and FOLFIRI, potentially improving responses in third-line treatment of mCRC.

DECLARATIONS

Authors' contributions

Conceptualization, writing, data analysis and interpretation: Okechukwu CC, Gmeiner WH

Performed experiments and data generation: Okechukwu CC

Writing, review, and editing: Okechukwu CC, Gmeiner WH

Availability of data and materials

All datasets and materials generated in this study are available from the corresponding author upon reasonable request.

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Conflicts of interest

Gmeiner WH is an inventor on a patent application for CF10 in colon cancer and an Editorial Board member of the journal *Cancer Drug Resistance*. Gmeiner WH was not involved in any steps of editorial processing, notably including reviewer selection, manuscript handling, or decision making. The other author 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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Supplementary Materials

[Supplementary Materials](#)

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Identification of antibody-drug conjugate payloads that are substrates of ATP-binding cassette drug efflux transporters

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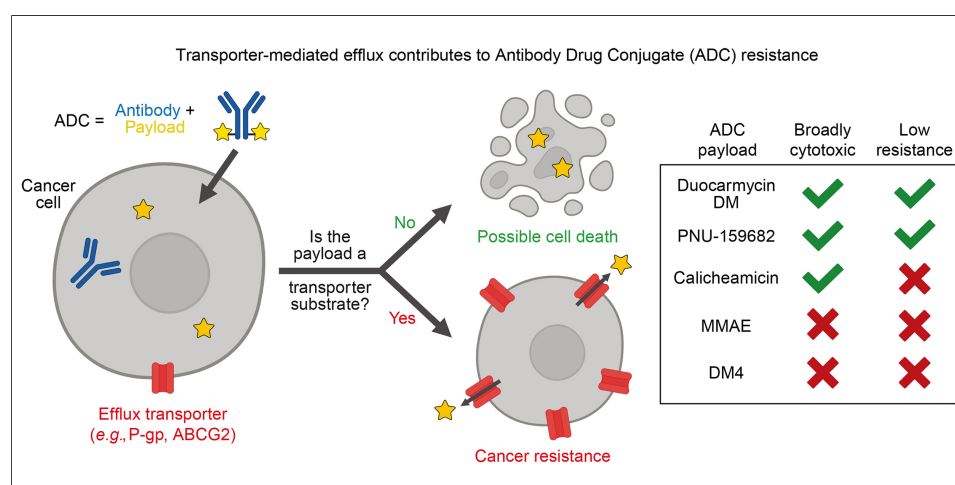
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Abstract

Aim: Antibody-drug conjugates (ADCs) feature an antibody recognizing a specific protein joined to a potent toxic payload. Numerous ADCs have received U.S. Food and Drug Administration (FDA) approval; however, clinical resistance arises. Resistance mechanisms include decreased expression or mutation of the antibody target, impaired payload release, or increased expression of adenosine triphosphate (ATP)-binding cassette (ABC) efflux transporters associated with multidrug resistance. We therefore sought to characterize the interactions of ABC multidrug transporters with ADC payloads.

Methods: We performed a high-throughput screen with 27 common ADC payloads using cell lines expressing ABC transporters P-glycoprotein [P-gp, encoded by ABC subfamily B member 1 (ABCB1)] or ABC subfamily B member G2 (ABCG2, encoded by ABCG2). Confirmatory assays were also performed using cells transfected to express P-gp, ABCG2, or multidrug resistance-associated protein 1 (MRP1, encoded by ABCC1).

Results: Several commonly used ADC payloads were substrates of P-gp, including



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calicheamicin γ 1, monomethyl auristatin E, mertansine (DM1), and ravtansine (DM4). All the pyrrolobenzodiazepines tested - SJG136, SGD-1882, SG2057, and SG3199 - were substrates of P-gp, ABCG2, and MRP1. The modified anthracyclines nemorubicin and its metabolite PNU-159682 were poorly transported by both ABCB1 and ABCG2 and displayed nanomolar to picomolar toxicity. Further, we found that the efficacy of the FDA-approved ADC mirvetuximab soravtansine, with DM4 as the toxic payload, was decreased in cell lines expressing P-gp. In contrast, Duocarmycin DM and PNU-159682 were exquisitely toxic to a panel of 99 cancer cell lines of varying origins.

Conclusion: Several commonly used ADC payloads can be transported by ABC transporters, potentially leading to transporter-mediated drug resistance in patients. Future ADCs should be developed using payloads that are not ABC transporter substrates.

INTRODUCTION

Antibody-drug conjugates (ADCs) have garnered significant attention as a strategy for selectively targeting tumor cells. ADCs consist of an antibody that targets a specific cell-surface antigen, conjugated to a small-molecule toxin (payload) via a cleavable linker^[1]. Following systemic distribution and binding to the antigen, the ADC is internalized and processed within the target cell lysosomes, releasing the toxin to exert its cytotoxic effects. Thus, provided the antibody targets a protein or epitope that is selectively expressed on the extracellular surface of cancer cells and minimally on other cell types in the body, the ADC should selectively kill tumor cells^[2]. The first ADC was granted U.S. Food and Drug Administration (FDA) approval in 2000 for the treatment of acute myeloid leukemia (AML); gemtuzumab ozogamicin targets cluster of differentiation 33 (CD33) and carries N-acetyl-calicheamicin γ 1 as the cytotoxic payload^[3]. Since then, several other ADCs have been approved for the treatment of cancer including brentuximab vedotin for Hodgkin lymphoma^[4], trastuzumab emtansine for breast cancer^[5], trastuzumab deruxtecan for breast cancer^[6] [recently approved for any human epidermal growth factor receptor 2 (HER2)-positive solid cancer^[7]], loncastuximab tesirine for diffuse large B-cell lymphoma^[8], enfortumab vedotin for urothelial cancers^[9] and mirvetuximab soravtansine (MIRV) for cisplatin-resistant ovarian cancers^[10]. FDA-approved ADCs, along with ADCs in clinical trials, have employed highly cytotoxic natural product-derived payloads that cause cell death via various modalities including induction of DNA damage, disruption of microtubules, and inhibition of topoisomerase^[11]. Many of these agents originate from molecules that were explored as small-molecule (untargeted) chemotherapeutics, but potent cytotoxic side effects led to their abandonment. A partial listing of approved ADCs and ADCs currently in clinical trials is included in [Table 1](#).

Despite responses of some cancers to ADCs, resistance to treatment is observed in the clinic. Clinically observed mechanisms of resistance include downregulation or mutation of the ADC antibody target, failure of lysosomes to release the payload, activation of alternative survival pathways, and the overexpression of efflux transporters^[12-16]. Patients whose leukemic blasts had lower levels of CD33 were found to respond more poorly to treatment with gemtuzumab ozogamicin *vs.* patients with blasts expressing higher levels^[17]. Similarly, patients whose tumors were found to express a truncated form of HER2 that does not bind to trastuzumab, p95HER2, were found to be largely resistant to trastuzumab emtansine^[18]. Decreased payload release in patient-derived samples has been linked to drug accumulation in the lysosomes due to increased lysosomal pH^[19]. Finally, overexpression of the adenosine triphosphate-binding cassette B1 (*ABCB1*) gene - which encodes the adenosine triphosphate (ATP)-binding cassette (ABC) transporter P-glycoprotein (P-gp) - has been inversely correlated with response to gemtuzumab ozogamicin in patients with AML^[17,20]. While individual reports of ABC transporter-mediated resistance of individual ADCs exist^[21,22], no systematic examination of the relationship between toxin sensitivity and ABC transporter expression has been reported.

Table 1. Approved and clinical candidate ADC small-molecule payloads

Name	Brand name	Payload	Linker	FDA status
Gemtuzumab ozogamicin	Mylotarg	Calicheamicin	Cleavable	Approved
Inotuzumab ozogamicin	Besponsa	Calicheamicin	Cleavable	Approved
Trastuzumab deruxtecan	Enhertu	Dxd (exatecan derivative)	Cleavable	Approved
Datopotamab deruxtecan	Datroway	Dxd (exatecan derivative)	Cleavable	Approved
Ado-trastuzumab emtansine	Kadcyla	DM1 (maytansinoid analog)	Non-cleavable	Approved
Mirvetuximab soravtansine	Elahere	DM4 (maytansinoid analog)	Cleavable	Approved
Brentuximab vedotin	Adcetris	MMAE	Cleavable	Approved
Polatuzumab vedotin	Polivy	MMAE	Cleavable	Approved
Enfortumab vedotin	Padcev	MMAE	Cleavable	Approved
Tisotumab vedotin	Tivdak	MMAE	Cleavable	Approved
Telisotuzumab vedotin	Emrelis	MMAE	Cleavable	Approved
Loncastuximab tesirine	Zynlonta	SG3199 (pyrrolobenzodiazepine dimer)	Cleavable	Approved
Sacituzumab govitecan	Trodelvy	SN-38 (active metabolite of irinotecan)	Cleavable	Approved
Disitamab vedotin	N/A	MMAE	Cleavable	In clinical trials
Depatuxizumab mafodotin	N/A	MMAF	Non-cleavable	In clinical trials
Zilovetamab vedotin	N/A	MMAE	Cleavable	In clinical trials
Vadastuximab talirine	N/A	SGD-1882 (pyrrolobenzodiazepine dimer)	Cleavable	In clinical trials
Camidanlumab tesirine	N/A	SG3199 (pyrrolobenzodiazepine dimer)	Cleavable	In clinical trials
Trastuzumab duocarmazine	N/A	Duocarmycin (Seco-DUBA)	Cleavable	In clinical trials
Pivekimab sunirine	N/A	DGN549 (Indolinobenzodiazepine pseudodimer)	Cleavable	In clinical trials
Belantamab mafodotin	Blenrep	MMAF	Non-Cleavable	Withdrawn

ADC: Antibody-drug conjugate; FDA: U.S. Food and Drug Administration; DM1: mertansine; DM4: raptansine; MMAE: monomethyl auristatin E; N/A: not available; MMAF: monomethyl auristatin F.

The objective of this study was to determine whether several commonly used ADC payloads were substrates for transport by ABC multidrug transporters with the goal of identifying cytotoxic agents that were not subject to transport out of the cell. P-gp substrates were identified by comparing compound activity between paired sets of low-expressing (sensitive) and over-expressing (resistant) cell lines for both P-gp and ABC subfamily G member 2 (ABCG2). Four paired cell sets were utilized - a drug-selected and transfected set for each transporter. Hits were confirmed in cells transfected to express P-gp, ABCG2, or multidrug resistance-associated protein 1 (MRP1, encoded by *ABCC1*). These results should aid investigators in designing ADCs that are not subject to ABC transporter-mediated resistance.

METHODS

Cell lines

HEK-293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and transfected with empty vector (pcDNA) or vector containing the human *ABCB1* (MDR-19), *ABCG2* (R-5), or *ABCC1* gene (MRP1) and were grown in Eagle's Minimum Essential Medium (EMEM) with 2 mg/mL Geneticin (G418) added to maintain transporter expression^[23]. The HeLa derivative KB-3-1 was purchased from ATCC and the P-gp overexpressing KB-8-5-11 subline was generated by selection with 100 ng/mL colchicine. The lines were maintained in Dulbecco's Modified Eagle Medium (DMEM); KB-8-5-11 additionally received 100 ng/mL colchicine^[24]. OVCAR8, NCI-ADR-RES (doxorubicin-selected, P-gp expressing OVCAR8 cell line), and H460 parental cells were obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute at Frederick, MD, and were cultured

in RPMI. The ABCG2-expressing subline H460 MX20 was generated by selecting H460 cells in 20 nM mitoxantrone^[25]. Media for all cell lines above was supplemented with 10% fetal bovine serum (FBS) and 1x penicillin-streptomycin. We also performed screening against a panel of 99 cancer cell lines; cell line source and culture conditions are reported in [Supplementary Table 1](#).

High-throughput screen

We explored 27 common ADC payloads, listed in [Table 2](#), in two orthogonal, paired cell line sets - a drug-selected and transfected set for each transporter - to identify substrates of the ABC transporters P-gp and ABCG2. To broadly assess ADC payload toxicity, we tested several cell lines of various origins that were acquired and grown in conditions reported in [Supplementary Table 1](#). As noted in [Table 2](#), compounds were sourced from Levena Biopharma (San Diego, CA), Selleck Chem (Houston, TX), ChemieTek (Indianapolis, IN), or Microsource Discovery Systems (Gaylordsville, CT). Screening was performed as previously described^[26]. Briefly, cells were plated at a density of 500 cells/well in 1536-well plates, with compounds added using a 1536-head pin tool (Kalypsys, San Diego, CA). Cells were incubated with cytotoxic drugs in the presence or absence of known inhibitors of the P-gp and ABCG2 pumps, tariquidar (TQR; 1 μ M) and Ko143 (5 μ M) respectively. Inhibition of efflux can restore intracellular accumulation; thus, re-sensitization of resistant cells confirms cytotoxic compounds are transporter substrates. Cells were incubated with drugs at various concentrations and processed with CellTiterGlo reagent (Promega, Madison, WI) to determine viability after 48 h. P-gp substrates were identified by comparing compound activity between paired cell line sets of low (sensitive) and high (resistant) transporter expression.

Confirmatory experiments on screen hits

Three-day cytotoxicity assays were performed using various payloads including other members of the pyrrolobenzodiazepine dimer (PBD) class, as well as the ADC MIRV (obtained from MedChemExpress, Monmouth Junction, NJ), with pcDNA (empty vector transfected), MDR-19 (*ABCB1* transfected), R-5 (*ABCG2* transfected) and MRP1 (*ABCC1* transfected) cells. Briefly, cells were plated (5,000 cells/well) in 96-well plates and allowed to attach overnight, after which the desired payloads or the ADC at the indicated concentrations were added and incubated with cells for three days. CellTiter Glo reagent (Promega) was then used according to the manufacturer's directions to assess GI_{50} (half-maximal growth inhibitory concentration) values.

Data analysis

To evaluate compound activity in the screening experiments, concentration-response curves (CRCs) were generated for each sample by plotting normalized response data against compound concentration. These curves were modeled using a four-parameter logistic regression, which provided key pharmacological parameters such as the half-maximal inhibitory concentration (IC_{50}) and maximal response (efficacy)^[27]. While many compounds showed well-defined, sigmoidal dose-response behavior with both upper and lower asymptotes, others displayed atypical or poor-quality CRCs, including shallow slopes, incomplete asymptotes, or responses derived from only a single active concentration point. Such cases were classified as low-confidence actives due to limited or ambiguous activity profiles. Furthermore, a compound's area under the curve (AUC) - calculated based on the screening data analysis and curve fittings - served as an integrated measure of compound activity, capturing both potency and efficacy. It was utilized for direct comparison of activity outcome across different cell lines and experimental conditions^[26].

RESULTS

A high-throughput screen identifies ADC payloads as substrates of P-gp and ABCG2

We evaluated 27 common ADC payloads in two orthogonal, paired cell sets to identify substrates of the ABC transporters P-gp and ABCG2 [[Table 2](#)]. Cytotoxic payloads were tested against two pairs of cell lines, one parental and one transporter-expressing line. For P-gp, the pairs were: (1) the parental KB 3-1 human

Table 2. ADC payloads tested in the HTS

Compound	Vendor	Mechanism of action
α -Amanitin	Levena Biopharma	RNA polymerase inhibitor
Ansamitocin P3	Levena Biopharma	Microtubule disruptor
Auristatin E	Levena Biopharma	Microtubule disruptor
Auristatin F	Levena Biopharma	Microtubule disruptor
Calicheamicin γ 1	Levena Biopharma	DNA damage
Camptothecin	Selleck Chem	Topoisomerase inhibition
Dasatinib	Selleck Chem	Tyrosine kinase inhibitor
DM1	Levena Biopharma	Microtubule disruptor
DM4	Levena Biopharma	Microtubule disruptor
Dolastatin 10	Levena Biopharma	Microtubule disruptor
Doxorubicin	Selleck Chem	Topoisomerase inhibition
Duocarmycin DM	Levena Biopharma	DNA alkylation
Duocarmycin MA	Levena Biopharma	DNA alkylation
Duocarmycin SA	Levena Biopharma	DNA alkylation
Duocarmycin TA	Levena Biopharma	DNA alkylation
Maytansinol	Levena Biopharma	Microtubule disruptor
MMAE	Levena Biopharma	Microtubule disruptor
MMAF	Levena Biopharma	Microtubule disruptor
Monomethyl dolastatin 10	Levena Biopharma	Microtubule disruptor
N-acetyl-calicheamicin γ 1	Levena Biopharma	DNA damage
Nemorubicin	Levena Biopharma	Topoisomerase inhibition
PBD Dimer (SGD-1882)	Levena Biopharma	DNA damage
PNU-159682	Levena Biopharma	Topoisomerase inhibition
SN-38	ChemieTek	Topoisomerase inhibition
Tubulysin IM-2	Levena Biopharma	Microtubule disruptor
Tubulysin M	Levena Biopharma	Microtubule disruptor
Vinblastine sulfate	Microsource Discovery	Microtubule disruptor

ADC: Antibody-drug conjugate; HTS: high-throughput screen; DM1: mertansine; DM4: ravtansine; MMAE: monomethyl auristatin E; MMAF: monomethyl auristatin F.

adenocarcinoma cell line and its colchicine-selected, P-gp-overexpressing sub-line KB 8-5-11; and (2) the HEK pcDNA human embryonic kidney cell line (transfected with an empty vector plasmid control) and its *ABCB1* stably transfected sub-line MDR-19. For *ABCG2*, the pairs were: (1) the parental H460 human lung carcinoma cell line and its mitoxantrone-selected, *ABCG2*-overexpressing sub-line H460 MX20; and (2) the same HEK pcDNA parent cell line as a comparator for the *ABCG2*-transfected HEK cell line, R-5. Transporter-specific efflux was demonstrated by testing all toxins for sensitization of transporter-expressing cells in the presence of either the P-gp inhibitor TQR or the *ABCG2* inhibitor Ko143. As such, six cell line conditions were used to evaluate toxins as P-gp substrates, and six cell line conditions were also used for evaluation of *ABCG2* substrates.

AUC values were calculated for each cell line with all payloads in the presence or absence of a specific inhibitor as previously described^[26] and are displayed in the heat maps in Figure 1. In the heatmaps, red denotes high cell death and thus a more potent compound, while blue denotes less cell death. In Figure 1A, cells expressing P-gp, when compared to parental cells, were found to be less sensitive (i.e., resistant) to

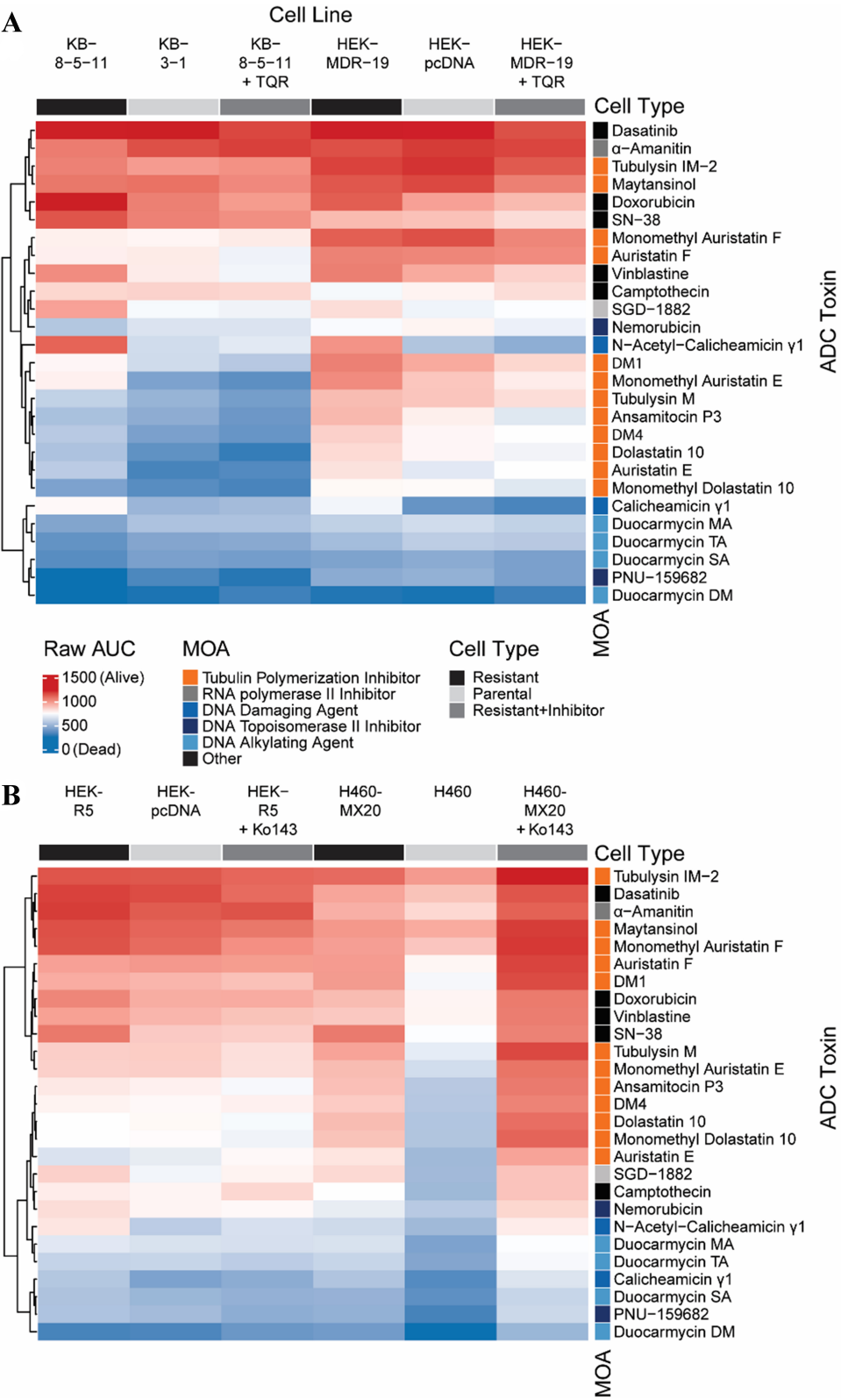


Figure 1. Heatmap of ADC payload activity across multidrug-resistant and control cell lines. (A) P-gp efflux potential: Unsupervised clustering of AUC values from dose-response viability assays of a panel of cytotoxic agents tested against parental, multidrug-resistant, and genetically modified KB and MDR-19 cell lines. Cell lines include KB-3-1 (parental) and KB-8-5-11 (P-gp overexpressing); HEK-293-derived MDR-19 (*ABCB1* transfected) and its vector control (pcDNA), as well as P-gp-inhibited counterparts (+TQR, tariquidar); (B) ABCG2 efflux potential: Unsupervised clustering of AUC values from dose-response viability assays of a panel of cytotoxic agents tested against parental, genetically modified, and multidrug-resistant HEK and H460 cell lines. Cell lines include H460 (parental) and H460-MX20 (ABCG2 overexpressing); HEK-derived HEK-R5 (ABCG2 transfected) and its vector control (pcDNA), as well as ABCG2-inhibited counterparts (+Ko143). ADC: Antibody-drug conjugate; P-gp: P-glycoprotein; AUC: area under the curve; *ABCB1*: adenosine triphosphate-binding cassette B1; TQR: tariquidar; MOA: mechanism of action.

several ADC payloads, including calicheamicin γ 1, dolostatin 10, monomethyl auristatin E (MMAE), tubulysin M, mertansine (DM1), and ravtansine (DM4). When the P-gp positive lines were incubated with the inhibitor TQR, resistance to calicheamicin γ 1, dolostatin 10, MMAE, tubulysin M, DM1 and DM4 was reversed. In the [Figure 1A](#) heat map, grouping the parental cell line next to the resistant cells plus efflux inhibitor highlights that the KB 8-5-11 line treated with TQR exhibited sensitivities similar to the parental KB-3-1 line; the MDR-19 line with TQR resembled the pcDNA-transfected cells, whereas the P-gp-expressing cells showed a distinctly different response. The cytotoxicity of nemorubicin, its metabolite PNU-159682 and the duocarmycin compounds appeared to be unaffected by P-gp levels.

As shown in [Figure 1B](#), few compounds appeared to be substrates of ABCG2, with the known substrate SN-38 being the most prominent compound that was subject to transport. Addition of the ABCG2 inhibitor Ko143 did not appear to re-sensitize the resistant cells, as R-5 cells with Ko143 did not resemble the sensitivity of pcDNA-transfected cells, and H460 MX20 cells with Ko143 responded similarly to H460 MX20 cells, rather than with the corresponding parental cell line. In line with efflux by ABCG2, the addition of Ko143 reversed resistance to SN-38.

To cross-validate efflux by the transporter of interest, we calculated and compared differences in AUC (deltaAUC) values between the parental and resistant lines and between the resistant lines in the absence or presence of inhibitor, as previously described [[Supplementary Figure 1A](#)]^[26]. Comparing deltaAUC values between the pcDNA/MDR-19 and MDR-19 + TQR pairs and the KB 3-1/KB 8-5-11 and KB 8-5-11 + TQR pairs, we found good correlation, with r^2 values of 0.72 and 0.92, respectively [[Supplementary Figure 1B](#)]. The results with P-gp contrasted with those of ABCG2, as few compounds were identified as ABCG2 substrates; the deltaAUC values for the pcDNA/R-5 and R-5 + Ko143/R-5 pairs and the H460/H460MX20 and H460 + Ko143/H460MX20 pairs showed poor correlation, with r^2 values of 0.32 and 0.31, respectively [[Supplementary Figure 1C](#)]. Thus, more payloads appeared to be transported by P-gp than ABCG2.

Validation of high-throughput screen hits

We validated selected screening results, adding a cell line (MRP1) developed by transfecting HEK293 cells with a plasmid containing the *ABCC1* gene [[Figure 2](#) and [Table 3](#)]. Consistent with data from the initial screen, MMAE, DM4, calicheamicin γ 1, and DM1 were confirmed as avid substrates of P-gp and we noted a small degree of resistance conferred to these payloads by ABCG2 [[Figure 2](#)]. Monomethyl auristatin F (MMAF) was not a substrate of any of the transporters, although it was less potent than the related compound MMAE [[Table 3](#)]. We also confirmed that nemorubicin and PNU-159682 were not substrates of any of the transporters examined, making these better ADC payloads; PNU-159682 is exquisitely toxic, with GI_{50} values in the picomolar range [[Table 3](#)].

Overexpression of ABCG2 conferred about 10-fold resistance to the camptothecin derivative exatecan, much less than the 96-fold resistance for SN-38 we previously reported for ABCG2-transfected cells^[28]. This is consistent with previous reports demonstrating that ABCG2 confers less resistance to exatecan compared to other camptothecin derivatives that are ABCG2 substrates^[29]. However, the exatecan derivative Dxd (deruxtecan) was readily transported by ABCG2 and was also a substrate for P-gp [[Figure 2](#) and [Table 3](#)].

As our screen originally contained only one member of the PBD dimer family (SGD-1882), we expanded the number of PBD dimer payloads examined. We found that HEK293 cells expressing any of the three transporters conferred some resistance to all PBD dimers examined: SG3199, SGD-1882, SJG136 (also known as SG2000), and SG2057. P-gp overexpression was found to confer the highest levels of resistance, followed by ABCG2 and finally MRP1 [[Figure 2](#) and [Table 3](#)].

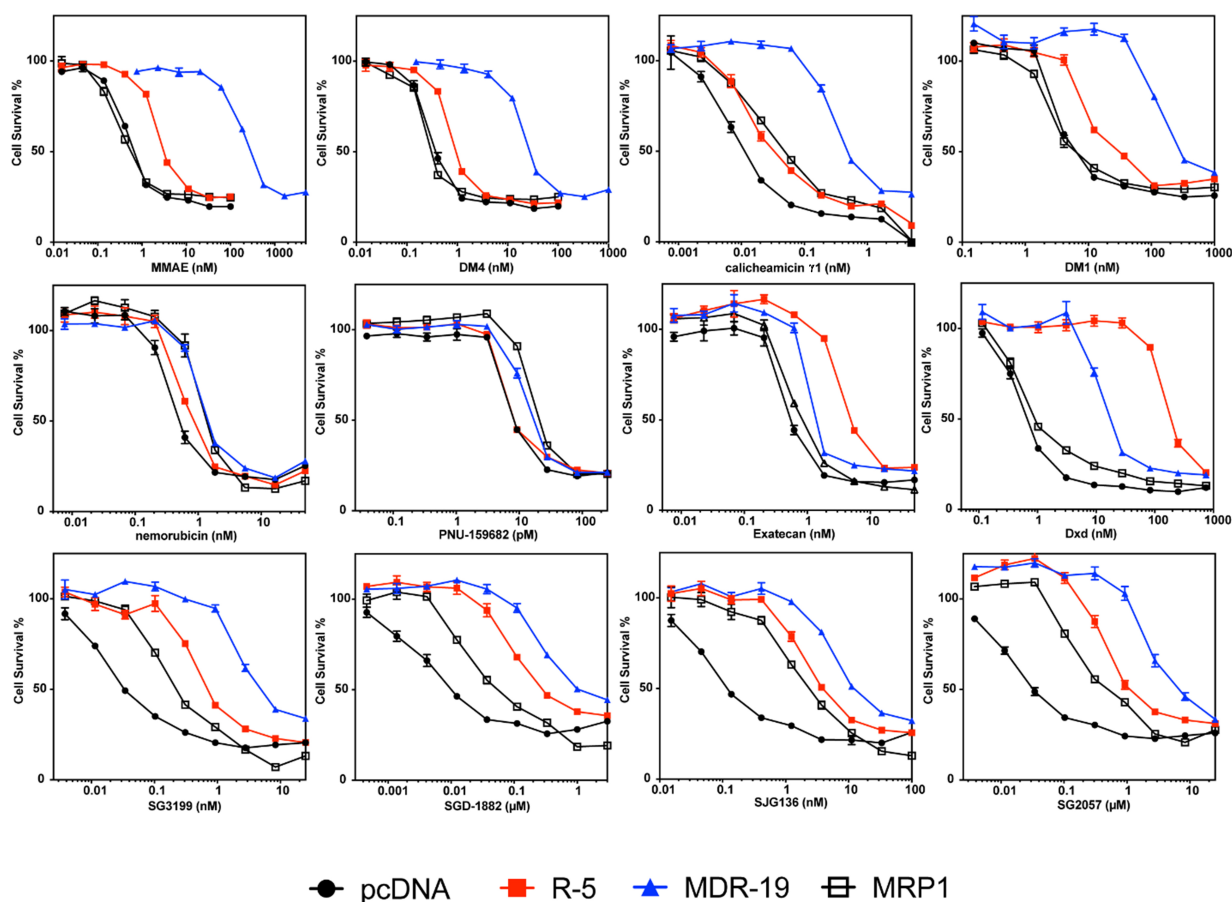


Figure 2. Confirmatory cytotoxicity assays with screen hits and additional ADC payloads. Three-day cytotoxicity assays were performed with the noted compounds as described in the Materials and Methods using HEK-293 cells transfected with empty vector (pcDNA, black dot), or vectors containing *ABCG2* (R5, red square), *ABCB1* (MDR-19, blue triangle), or *ABCC1* (MRP1, black box). Results from one of three independent experiments are shown. Cytotoxicity data are summarized in Table 3. ADC: Antibody-drug conjugate; *ABCG2*: ABC subfamily G member 2; *ABCB1*: adenosine triphosphate-binding cassette B1; MRP1: multidrug resistance-associated protein 1; MMAE: monomethyl auristatin E; DM4: ravidansine; DM1: mertansine.

P-gp overexpression confers resistance to MIRV

The screening approach described here evaluated small-molecule cytotoxic drugs employed as payloads in experimental and therapeutic ADCs. Examining how drug transporter-mediated susceptibility confers resistance to an ADC (where the toxin is liberated from the antibody within the cell and subsequently effluxed) is important for extrapolating our results. We thus tested the effect of P-gp expression on the sensitivity of cultured cells to an ADC. MIRV is an ADC that was approved by the FDA in 2022 for the treatment of folate receptor alpha-positive, platinum-resistant ovarian, fallopian tube or peritoneal cancer^[10]. The toxic payload is DM4, which we found to be a P-gp substrate in our screen. Testing MIRV with the KB3-1/KB-8-5-11 pair used in our screening assay and the OVCAR8/NCI-ADR-RES cell line pair, we found that the P-gp overexpressing lines KB 8-5-11 and NCI-ADR-RES were quite resistant to treatment with MIRV compared to the parental lines [Figure 3 and Supplementary Figure 2]. These results confirm that screening of isolated payloads for susceptibility to P-gp efflux translates to cellular resistance to an ADC carrying a P-gp-substrate toxin, and suggest that P-gp may contribute to clinical resistance to MIRV.

Duocarmycins and PNU-159682 are highly toxic to a broad range of cancer cell lines

We next explored the toxicity of the various payloads across a broad range of cell lines, including lines from cancers originating from the skin, kidney, pancreas, lung and blood. We excluded five payloads from our original list of 27 - vinblastine, SN-38, camptothecin, dasatinib and doxorubicin - as these compounds have

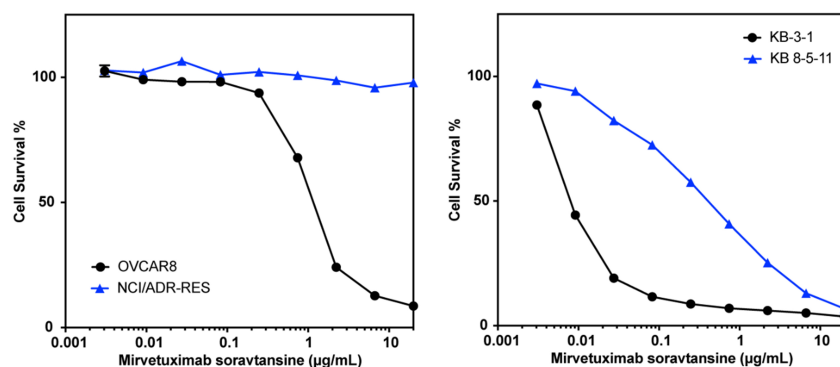


Figure 3. P-gp overexpression confers resistance to treatment with MIRV. Three-day cytotoxicity assays were performed with MIRV as described in the Materials and Methods using the OVCAR8/NCI-ADR-RES pair (left) or the KB 3-1/KB 8-5-11 pair (right). Results from one of two independent experiments are shown. P-gp: P-glycoprotein; MIRV: mirvetuximab soravtansine.

Table 3. Cross-resistance profile to select ADC payloads in cells expressing P-gp, ABCG2 or MRP1

Drug	pcDNA	ABCB1		ABCG2		ABCC1	
		MDR19	RR	R5	RR	MRP1	RR
DM1 (nM)	7.8 ± 1.4	410 ± 120	52	35 ± 4.1	4.5	9.5 ± 3.7	1.2
DM4 (nM)	0.43 ± 0.075	42 ± 20	96	1.4 ± 0.49	3.3	0.58 ± 0.37	1.3
MMAE (nM)	0.90 ± 0.23	360 ± 110	400	5.1 ± 1.9	5.7	1.2 ± 0.80	1.3
MMAF (μM)	0.99 ± 0.097	2.0 ± 1.7	2.0	0.99 ± 0.097	1.0	1.0 ± 0.39	1.0
Calicheamicin γ1 (nM)	0.010 ± 0.0042	0.73 ± 0.41	71	0.029 ± 0.0047	2.9	0.028 ± 0.017	2.7
Exatecan (nM)	0.59 ± 0.19	1.4 ± 0.15	2.4	5.0 ± 0.80	8.5	1.0 ± 0.19	1.8
Deruxtecan (nM)	0.82 ± 0.18	17 ± 1.2	20	190 ± 29	240	1.1 ± 0.40	1.4
Nemorubicin (nM)	0.76 ± 0.31	1.5 ± 0.15	2.0	1.1 ± 0.27	1.4	1.6 ± 0.21	2.2
PNU-159682 (pM)	13 ± 9.6	16 ± 3.7	1.2	13 ± 8.4	1.0	19 ± 8.6	1.4
SJG136 (nM)	0.098 ± 0.019	16 ± 6.2	170	4.6 ± 0.68	47	1.2 ± 1.1	12
SGD-1882 (nM)	0.0066 ± 0.0028	0.85 ± 0.34	130	0.22 ± 0.093	33	0.095 ± 0.036	14
SG2057 (nM)	0.025 ± 0.0087	5.7 ± 1.2	230	0.73 ± 0.34	29	0.27 ± 0.20	11
SG3199 (nM)	0.045 ± 0.011	5.6 ± 1.6	120	0.90 ± 0.31	20	0.21 ± 0.026	4.6

Results are mean GI₅₀ values +/- standard deviation. The RR value was computed by dividing the GI₅₀ value for the lines expressing the ABC transporters by the empty vector (pcDNA) line. Results are from three independent experiments. ADC: Antibody-drug conjugate; P-gp: P-glycoprotein; ABCG2: ABC subfamily G member 2; MRP1: multidrug resistance-associated protein 1; ABCB1: adenosine triphosphate-binding cassette B1; RR: relative resistance; DM1: mertansine; DM4: ravtansine; MMAE: monomethyl auristatin E; MMAF: monomethyl auristatin F; ABC: adenosine triphosphate (ATP)-binding cassette.

been well characterized. As shown in Figure 4, hematologic cancers were sensitive to nearly all the compounds tested, while lung and pancreatic cancers tended to be less sensitive. The duocarmycins and PNU-159682 were uniformly toxic across the cell line panel. Given that the duocarmycins and PNU-159682 were not found to be substrates of ABC transporters, these compounds should be considered for use as ADC payloads in the future.

DISCUSSION

We systematically screened 27 commonly used ADC payloads [Table 2] in two orthogonal, paired cell sets to identify substrates of the ABC transporters P-gp and ABCG2. Of the studied DNA-damaging agents, we identified calicheamicin analogs, a PBD dimer (SGD-1882), and doxorubicin to be substrates of the ABC transporter P-gp. Of the tubulin-targeting agents, vinblastine and all auristatin and maytansinoid derivatives

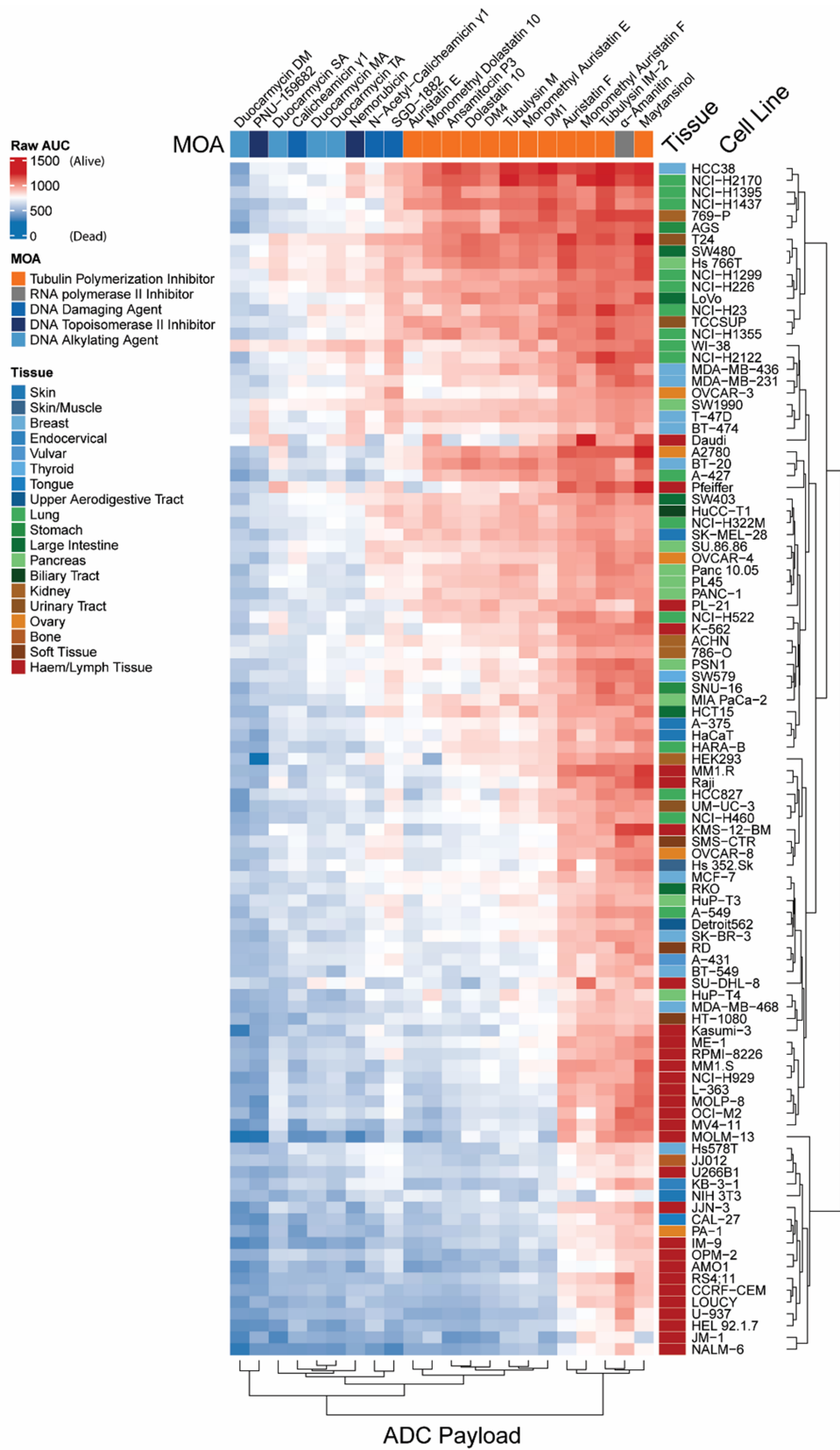


Figure 4. Differential cytotoxicity of ADC payloads across cancer cell lines from diverse tissue origins. Heatmap showing AUC values from dose–response viability assays for a panel of ADC payload compounds tested across a large set of human cancer cell lines. Each row represents a unique cancer cell line, annotated by tissue of origin (colored bar on the right-hand side), and each column corresponds to an ADC payload compound, annotated by mechanism of action on the top of the heatmap. AUC values are color-coded, with red indicating higher AUC (lower sensitivity; more resistant) and blue indicating lower AUC (higher sensitivity; more cytotoxic effect). Clustering (both rows and columns) was performed using hierarchical methods based on compound response profiles. ADC: Antibody-drug conjugate; AUC: area under the curve; MOA: mechanism of action.

were strong P-gp substrates, whereas MMAF was less susceptible to transport. SN-38 was identified as a substrate of ABCG2; however, no other cytotoxic payloads of the 27 payloads that were screened were found to be transported by ABCG2. In confirmatory studies with cells transfected with plasmids containing *ABCB1*, *ABCC1* or *ABCG2*, cells expressing P-gp were resistant to MMAE, DM1, DM4 and calicheamicin γ 1. Cells expressing ABCG2 were resistant to the camptothecin derivatives exatecan and Dxd, while P-gp overexpression also conferred resistance to Dxd. Cells expressing P-gp, MRP1 or ABCG2 were resistant to all PBD dimers tested. Notably, none of the transporters conferred resistance to the duocarmycin analogs, camptothecin, nemorubicin, PNU-159682, and α -amanitin. Additionally, the duocarmycins, nemorubicin, and PNU-159682 were toxic to a wide range of cancer cell line models, suggesting their potential value for further development as ADC payloads.

Our results are in agreement with earlier *in vitro* studies using cell lines and *in vivo* mouse models which have demonstrated that overexpression of ABC transporters can drive resistance to some ADCs. Naito *et al.* found that leukemia cell lines that overexpressed P-gp were resistant to treatment with gemtuzumab ozogamicin and that combination with P-gp inhibitors such as valspodar or biricodar reversed resistance^[30]. In the MRP1-overexpressing cell line HL-60/ADR, addition of the MRP1 inhibitor MK-571 was found to increase sensitivity to gemtuzumab ozogamicin, suggesting that MRP1 mediates resistance to the ADC^[31]; however, overexpression of ABCG2 does not appear to cause resistance^[32]. Studies using cell lines expressing P-gp, MRP1, or ABCG2 with the CD33-targeting ADC AVE9633 (payload DM4), showed that only P-gp could confer resistance^[21]. Repeated treatment of mice xenografted with the SUM190 breast cancer cell line with the ADC N41mab-vcMMAE (payload MMAE)-induced refractory tumors which were found to overexpress P-gp as the mechanism of resistance^[33]. An ADC targeting the delta-like non-canonical Notch ligand 1 protein (ADCT-701; payload SG3199) was found to be less effective in adrenocortical cancer cell lines and organoids with high expression of P-gp, and the free drug was also reported to be a substrate of P-gp^[34]. The ADC OBI-992 (payload exatecan) was shown to retain efficacy in cellular models overexpressing P-gp, but ABCG2 conferred modest resistance^[35,36]. Treatment of cells with both ADCs carrying SG3199 and SG3199 alone showed acquired resistance mediated by ABC transporters, with reversal of resistance by transporter siRNA or transporter inhibitors^[37]. Notably, xenograft tumors with acquired resistance to MMAE-containing ADCs driven by P-gp overexpression were sensitive to the identical antibody-linker when PNU-159682 replaced MMAE as the payload^[38]. The findings of these studies are consistent with our results, confirming that calicheamicin, DM4, MMAE, and SG3199 are substrates of P-gp and - given that PNU-159682 remains active in MMAE-resistant cells - suggesting that selecting payloads with low efflux potential may reduce the risk of acquired resistance.

Overexpression of P-gp has also emerged as a marker of resistance in a subset of patients who have been treated with gemtuzumab ozogamicin and brentuximab vedotin. Early studies examining leukemic blasts from patients with resistant disease demonstrated that P-gp and MRP1 expression could lead to resistance^[20,31,39]. P-gp expression was found to inversely correlate with clinical response to gemtuzumab ozogamicin in patients with AML^[17]. Similarly, a study examining a small cohort of lymphoma patients resistant to gemtuzumab vedotin reported P-gp overexpression in a patient with Hodgkin Lymphoma^[22]. Finally, a case report of a patient with bladder cancer whose disease had progressed after treatment with enfortumab vedotin reported high levels of P-gp in the resistant tumor^[40].

These initial studies suggested that the addition of a P-gp inhibitor might be beneficial to counter acquired resistance via P-gp overexpression, leading to clinical trials combining P-gp inhibitors with ADC treatment. The P-gp inhibitor zosuquidar was found to reverse resistance to gemtuzumab ozogamicin in *ex vivo* studies using P-gp-positive blasts obtained from patients with resistant disease^[41]. In a clinical trial combining zosuquidar with gemtuzumab ozogamicin, greater overall survival was noted in patients with P-gp-positive

resistant disease^[42]. A small clinical study combining cyclosporine with gemtuzumab ozogamicin in patients with resistant disease led to an increased overall and complete response rate^[43]. However, the final findings from a larger trial (NCT03013933) exploring the combination of brentuximab vedotin with cyclosporine were less positive, largely due to toxicity from the use of cyclosporine A as the P-gp inhibitor, highlighting the importance of inhibitor choice when designing combination trials^[44,45]. Beyond strategies for advancing future ADC development^[12], the addition of efflux inhibitors to overcome ADC resistance may offer opportunities to improve efficacy of currently approved ADCs.

We report duocarmycin analogs to be broadly cytotoxic and, in agreement with prior studies, not be substrates of P-gp or ABCG2, positioning them as improved ADC payloads^[46]. Optimization of duocarmycin molecules has yielded prodrugs that can be activated upon release from an ADC [e.g., seco-duocarmycin-hydroxybenzamide-azaindole (seco-DUBA)]^[47], with promising preclinical reports on ADC efficacy^[48,49]. Current clinical trials with Trastuzumab duocarmazine are testing the clinical efficacy of these developments to see if they yield less susceptibility to acquired resistance by payload efflux^[50].

Following localization to a target cell by the antibody, an ADC is internalized and the payload is released to exert its cytotoxic effect. Depending on the linker chemistry, the released species may retain a residual linker moiety with potential to alter its physicochemical properties and potential recognition by ABC transporters. For instance, DM1 alone is a substrate of P-gp, but DM1 released from Ado-trastuzumab emtansine is a charged metabolite, which may alter its efflux^[51]. While our study focused on isolated ADC payload molecules and MIRV, which contains a cleavable linkage that yields free DM4 without any remaining linker moiety, prior studies have reported acquired resistance to ADCs with varied linker chemistries.

While our data strongly support a role for ABC transporters in mediating resistance to ADCs via payload efflux, we cannot exclude additional resistance mechanisms. Our experiments used a focused set of *in vitro* cellular systems and specific ADC/payload chemotypes, which may not fully capture the diversity of resistance that arises in patients; other processes reported in the literature, such as altered target antigen expression, changes in ADC trafficking or processing, or adaptations in DNA-damage and cell-death pathways, may also modulate ADC sensitivity. Finally, although we characterize ABC transporter-mediated payload efflux *in vitro*, future studies in appropriate *in vivo* models and with longitudinal clinical samples are needed to define how ABC transporters influence acquired resistance in the clinical setting.

In conclusion, we show that the payloads of several FDA-approved ADCs are strong substrates of P-gp, suggesting that active transport and efflux of the released payload may contribute to acquired resistance to clinical ADCs. These effects may also increase exposure of the cytotoxic payload to systemic tissues and could contribute to *in vivo* off-target toxicities. We identified ADC payloads that were not substrates of either P-gp or ABCG2 - notably the duocarmycin series and PNU-159682 - which were among the most broadly cytotoxic of all toxins tested in a panel of 99 cancer cell lines - and suggest that these compounds be prioritized as future ADC payloads due to their potential for reduced susceptibility to transporter-mediated acquired resistance^[52].

DECLARATIONS

Authors' contributions

Data curation, formal analysis, investigation, visualization, writing - original draft preparation, writing - review and editing: Roth JS

Formal analysis, investigation, visualization: Guo H, Chen L

Formal analysis, investigation, visualization, writing - original draft preparation, writing - review and editing: Shen M

Data curation, formal analysis, investigation, writing - review and editing: Gbadagesin O

Data curation, visualization, investigation, writing - original draft preparation, writing - review and editing:

Robey RW

Supervision, conceptualization, writing - review and editing: Gottesman MM

Conceptualization, resources, supervision, writing - original draft preparation, writing - review and editing: Hall MD

Availability of data and materials

Data from the high-throughput screens has been submitted to the PubChem repository with AIDs 2061167 and 2202241. Data can be accessed via the links: <https://pubchem.ncbi.nlm.nih.gov/bioassay/2061167> and <https://pubchem.ncbi.nlm.nih.gov/bioassay/2202241>.

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Conflicts of interest

Gottesman MM and Robey RW are Editorial Board Members of *Cancer Drug Resistance*. They were not involved in any steps of the editorial process, notably including reviewers' selection, manuscript handling, or decision-making. The other 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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Supplementary Materials

[Supplementary Materials](#)

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