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Review

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Reactive oxygen species in the progression and treatment of malignant mesothelioma

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Abstract

Malignant mesothelioma (MM) is an aggressive cancer that affects the pleural and peritoneal mesothelial lining of the lungs and abdomen. Survival rates for patients with MM remain extremely low and effective treatments are limited. MM tumors harbor both genotypic and phenotypic features that indicate MM tumor cells are under increased oxidative stress, similar to other aggressive cancers. This increased oxidative stress in MM cells supports aggressive growth while providing a therapeutic vulnerability exploitable by redox-modulating compounds. MM tumor cells also exhibit altered mitochondrial structure and function that contribute to the disease through perturbations in metabolism and reactive oxygen species (ROS) production and metabolism. Targeting the altered redox status in cancer through increasing cellular ROS levels directly or inhibiting cellular antioxidant pathways and disrupting ROS scavenging mechanisms has become an exciting area for therapeutic intervention. This review discusses ROS sources and signaling, mitochondrial structure and function and targeting mitochondria ROS as a therapeutic approach for the treatment of MM.

Keywords: Malignant mesothelioma, targeting cellular redox status, pro-oxidant therapy

INTRODUCTIONS

Malignant mesothelioma (MM) is an aggressive form of cancer that primarily affects the pleural and

peritoneal mesothelial lining of the lungs and abdomen. Although asbestos exposure is a major risk factor for the development of MM, little is known about the etiology of the disease[1]. Similar to the majority of other solid tumors, MM is strongly linked to altered metabolism, changes in mitochondrial dynamics and an imbalance in the production and clearance of reactive oxygen species (ROS). Because of this, ROS production and metabolism have become an exciting target for cancer treatments[2] including MM[3]. Numerous approaches currently used in the clinic to treat MM, including cisplatin, have also been shown to modulate ROS levels[4]. Recently, thiostrepton (TS), an inhibitor of mitochondrial peroxiredoxin 3 that induces elevated ROS levels and cell death[5,6], entered phase 1/2 clinical trials (NCT05278975), providing a new and exciting redox-dependent therapy for the treatment of MM. This review will discuss the role of ROS in cancer development and the unique mitochondrial dynamics and redox status in MM that may be an effective target for anticancer therapies.

CANCER AND ROS

Ros in cell signaling

The role of ROS in normal and cancer cells is far more than just damaging or a byproduct of oxidative metabolism. ROS contribute to cell signaling cascades through a process termed "redox-dependent signaling"[7]. ROS can directly or indirectly oxidize cysteine residues in proteins[8] through hydrogen peroxide (H_2O_2)-mediated oxidation of target proteins or through peroxiredoxin (Prx)-dependent redox relays[9]. ROS are produced by intracellular and extracellular sources, including asbestos, and dynamically regulate numerous cell signaling pathways[10]. Intracellular sources of ROS, such as NADPH oxidases and the mitochondrial electron transport chain, participate in redox-dependent signaling spatially and temporally[11,12] [Figure 1]. Control over the amount, timing and location of ROS contributes to specific redox signaling events, akin to cellular control over protein phosphorylation cascades[13]. One well-known redox signaling event is in the cell's response to hypoxia, which is mediated by the stabilization of hypoxia-inducible factors (HIFs). Under normal conditions, prolyl hydroxylase domain protein 2 (PHD2) prevents HIF stabilization by hydroxylating two of its proline residues, marking it for degradation[14]. PHD2 is deactivated at low oxygen levels, allowing HIF stabilization. A study performed by Chandel *et al*. found that the production of mROS was required for HIF stabilization under hypoxia, though the mechanism is still unclear[15,16]. In cancer cells, HIF stabilization stimulates angiogenesis, glycolysis, and cell survival, key hallmarks of tumorigenesis[2].

ROS IN TUMORIGENESIS AND TUMOR CELL RESPONCE TO ROS

The increased production of ROS in tumor cells is described as a "double-edged sword" in the process of tumorigenesis[17]. Increased ROS, often driven by oncogene activation[18], must be managed by cancer cells by upregulating various antioxidant networks, as excessive oxidative stress would normally induce senescence and/or apoptosis in cells[19] [Figure 1]. Conversely, ROS is also thought to promote cell proliferation by inducing DNA mutations and activating redox-dependent signaling pathways[17]. One specific way ROS may promote tumorigenesis is by activating the phosphoinositide 3-kinase (PI3K) pathway. This pathway is upregulated in cancer cells and promotes cell proliferation, survival, and mobility[20]. It is also known that ROS inhibits phosphatase and tensin homolog (PTEN) activity, which allows for constitutive expression of PI3K when inactivated[21,22] [Figure 1]. Increased ROS levels in human MM cells enhance the expression of the oncogenic transcription factor FOXM1 which supports cell cycle progression and escape from oxidative stress[23-25] [Figure 1]. ROS also have the ability to alter metabolism, an example of which is by oxidation of key cysteine residues in pyruvate kinase M2 (PKM2). The oxidation of Cys^{358} on PKM2 is thought to increase pentose phosphate pathway flux and cell proliferation in hypoxic conditions[26]. Inhibition of PKM2 has been associated with increased tumorigenesis[27,28].

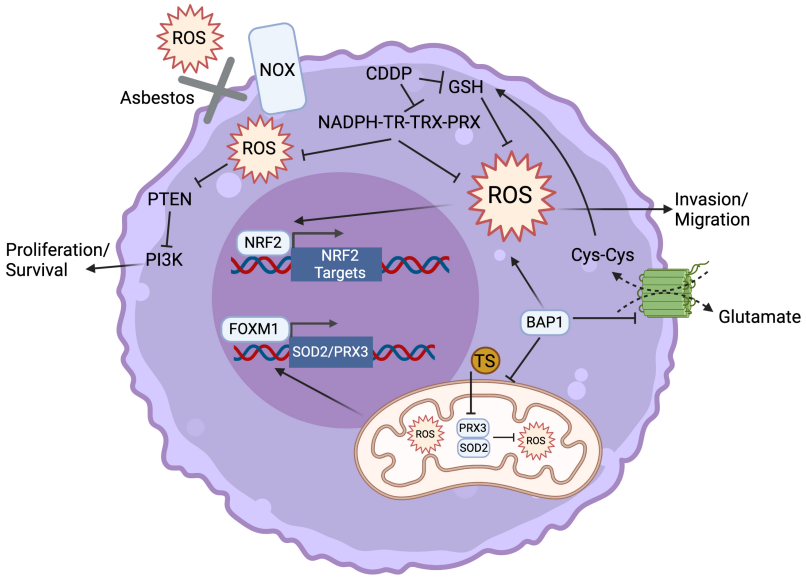

Figure 1. The redox landscape of MM tumors and methods of targeting ROS: ROS are primarily derived from asbestos fibers, NADPH Oxidases (NOXs) and mitochondria in MM tumor cells. ROS levels are balanced by the expression of ROS scavenging pathways, including the TR-TRX-PRX antioxidant axis, and increased GSH synthesis through Cystine (Cys-Cys) import via the SLC7A11 uniporter. FOXM1 and NRF2 are redox-responsive transcription factors that support ROS scavenging gene expression. BAP1 alters cellular redox status through downregulation of the SLC7A11 uniporter and disruption of mitochondrial bioenergetics. ROS inhibit PTEN phosphatase activity driving increased PI3K activity that supports tumor cell proliferation and survival. Cisplatin (CDDP) targets DNA, TRX and GSH which lead to increased ROS levels. Thiostrepton (TS) inhibits mitochondrial PRX3 which lead to increased mitochondrial ROS. High ROS levels are incompatible with cell survival.

Tumor cells must balance the increased levels of ROS associated with transformation and therefore acquire genetic and phenotypic features to survive under otherwise inhospitable redox conditions[29]. The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is considered to be the predominant regulator of antioxidant enzyme expression. NRF2 is activated under conditions of oxidative stress through a mechanism involving the redox-dependent release of KEAP1, allowing NRF2 translocation to the nucleus and regulation of antioxidant genes through antioxidant response element (ARE) binding[30]. NRF2 is responsible for producing and regenerating glutathione (GSH), a major antioxidant cofactor in the cell[31]. NRF2 also has a role in the production of NADPH, which is used to regenerate many antioxidant enzymes[17,32] including the thioredoxin reductase (TR) - thioredoxin (TRX) - peroxiredoxin (PRX) antioxidant network[33] [Figure 1].

The forkhead box (FOX) family of proteins also plays a significant role in tumor cell escape from oxidative stress. The FOX family of proteins plays an important role in cell proliferation, cell metabolism, stress responses, and aging[17]. FOXO specifically seems to regulate the expression of superoxide dismutases (SODs), catalase and sestrin 3 under conditions of oxidative stress[34]. FOXM1 expression is increased in H-RAS transformed cells and is required for balancing cellular ROS levels and escaping from oncogene-induced senescence[25]. FOXM1 expression is upregulated in MM and plays an important role in MM cell survival (see below). p53, a prominent tumor suppressor gene, is also thought to have a regulatory role in antioxidant gene expression, though its expression has been thought to produce both pro- and antioxidant responses[35]. It is thought to promote the production of glutamate which is required for GSH synthesis[36]. p53 also promotes sestrin 1 and 2 expression[37]. Experiments with mutated forms of p53 that prevent its ability to induce apoptosis and cell cycle arrest showed that it still retained a tumor suppressive ability[38]. This suggests that its function appears to be partly due to its ability to suppress ROS production[39].

Interestingly, p53, although mutated in many forms of cancer, is almost always a wild type in MM cell lines. Its function, however, is usually inhibited by other gene deletions frequently found in MM cells[40].

ROS IN MALIGNANT MESOTHELIOMA

Human MM cell lines demonstrate elevated levels of antioxidant enzymes such as manganese superoxide dismutase (MnSOD)[41], peroxiredoxins (PRXs) and the mitochondrial thioredoxin reductase 2 (TrxR2) - thioredoxin 2 (Trx2) - peroxiredoxin 3 (Prx3) antioxidant network[42] compared to normal mesothelial cells. The upregulation of MnSOD is significant as it is the only antioxidant induced by asbestos exposure[43] and contributes to the high resistance to oxidant chemotherapeutic drugs displayed by MM cells[44]. MM cells also display increased expression of many PRXs[45], a family of H_2O_2 scavenging enzymes found in the cytoplasm (PRX1, PRX2), mitochondria (PRX3, PRX5) and endoplasmic reticulum (PRX4). Some members have notable functions, such as PRX2, which induces cell proliferation and protects cells from undergoing oxidative stress-induced apoptosis[46]. PRX3 protects tumor cells from apoptosis[47] and knockdown of PRX3 in human MM cells leads to reduced proliferation and altered cell cycle progression[5,23]. The role of PRX3 in cancer is still being uncovered, but its importance in maintaining mitochondrial redox status is clear as PRX3 is estimated to metabolize ~90% of mitochondrial H_2O_2[48]. Targeting PRX3 for the treatment of MM is a new avenue being explored in clinical trials (elaborated below).

Disruption of cellular redox status via depletion of glutathione (GSH) and iron-dependent lipid oxidation contributes to a type of programmed cell death termed ferroptosis[49]. Inactivation of NF2, LATS1, or LATS2, genes involved in signaling cascades that prevent ferroptosis, are recently identified mutations in MM cells that sensitize them to ferroptosis[50]. Approaches to targeting these mutations and taking advantage of this ferroptosis sensitivity, as well as looking into other possible pathways involved in ferroptosis, are an avenue for therapeutic intervention in MM. One proposed target is BAP1, the most commonly mutated gene in MM, which can inhibit ferroptosis when mutated[51,52]. Interestingly, fibroblasts harboring heterozygous BAP1 mutations show altered metabolite profiles and mitochondrial respiration, including reduced citric acid cycle metabolites and mitochondrial oxygen consumption[53]. It was concluded that BAP1 mutant fibroblasts are preferentially undergoing aerobic glycolysis (Warburg effect). Additional studies in a mesothelioma cell line (NCI-H226), which does not express BAP1, showed that reconstitution of BAP1 into these cell lines promoted ROS production, gene-expression changes indicative of increased cellular oxidative stress and increased sensitivity to exogenous H_2O_2[54] [Figure 1]. These results provide a path forward for looking into BAP1 status in the context of redox-dependent therapies.

FOX protein family members, including FOXM1, are thought to mediate tumorigenesis and promote the survival of MM cells[24]. High FOXM1 expression is found in MM cells, and tumors and knockdown of FOXM1 with siRNA slows the growth of MM cells[55,56]. FOXM1 also regulates the expression of genes involved in cell survival and cell cycle progression. The two oncogenic isoforms of the protein, FOXM1B and FOXM1C, act as transcriptional activators and are both upregulated in MM cancer cells, suggesting that FOXM1 may be a viable target for cancer treatment[24,57]. The expression of FOXM1 is induced by the production of H_2O_2[25,58]. FOXM1 counteracts oxidative stress by upregulating the expression of antioxidant enzymes such as SODs, catalase, and PRX3, which suppresses oncogene-induced senescence and supports tumor cell growth[25] [Figure 1]. PRX3 functions by metabolizing H_2O_2, which causes catalytic cysteine residues in PRX3 to form a disulfide bond. This bond is reduced by thioredoxin 2 (TRX2), regenerating PRX3 to its active conformation and able to metabolize another molecule of H_2O_2[59]. TRX2 is subsequently regenerated by TR2 using NADPH as the reductant[60]. Notably, TR2 and TRX2 are also upregulated in cancer cells, and measuring the activity of TR2 is proposed to be a useful way to monitor the growth of MM cell lines[61]. The TR2-TRX2-PRX3 system has been identified as a pathway for therapeutic intervention in

preclinical MM cell and animals models, which will be discussed below.

DISRUPTION OF MITOCHONDRIAL DYNAMICS IN MALIGNANT MESOTHELIOMA

The mitochondria are thought of as a dynamic network that fuse (fusion) and divide (fission) in order to adapt to the metabolic needs of the cell[62]. The process of fission is mediated by the GTPases dynamin-related protein1 (Drp1) and dynamin 2 (Dnm2)[63]. Similarly, mitochondrial fusion is mediated by mitofusins Mfn1 and Mfn2 that help perform the fusion of two mitochondrial membranes[64]. In cancerous cells, the mitochondrial network is often fragmented, and redox-dependent signaling is believed to be key to these shape changes[65,66]. One of these pathways is hypoxia-induced mitochondrial fission, which occurs when HIFs induce Drp1 expression. In addition to mitochondrial fragmentation, HIF expression is linked to increased metastatic activity. This suggests that HIFs are an important mediator for both mitochondrial fission and cancer cell proliferation[67,68]. Another important mediator is the p38 mitogen-activated protein kinase (MAPK), which stimulates stress response pathways upon receiving redox signals[69]. One direct way is to phosphorylate Drp1 directly to induce mitochondrial fission[70].

The transcription factor nuclear factor κB (NF-κB), which plays a role in inflammation and is upregulated in cancer, is also thought to alter mitochondrial dynamics. Elevated NF-κB levels in cigarette smoke-induced mitochondrial fragmentation were found to correlate with increased expression of Drp1 and decreased expression of Mfn2[71]. More recent research has also shown that the principal component of that pathway is the NF-κB inducing kinase (NIK). In addition to having pro-fission activity, NIK was also shown to promote mitochondrial migration towards the cell periphery, which was shown to correlate with increased tumor invasiveness[72,73]. NF-κB activity is upregulated following asbestos exposure[74], and sustained NF-κB activity has been observed in MM cells[75]. Downregulation of NF-κB with Onconase decreased MM proliferation and invasion[76]. Although mitochondrial defects were not reported in these studies, the cellular responses observed warrant evaluating strategies targeting NF-κB with disruption of mitochondrial dynamics.

MM cells also appear to demonstrate a difference in mitochondrial morphologies. A study performed by Lennon *et al.* evaluated the fission/fusion rate of various MM cells using fractal dimension and lacunarity measurements to characterize mitochondrial architectures[77]. They found that MM cell lines displayed a low fractal dimension and high lacunarity compared to control mesothelial cells. The low fractal dimension and high lacunarity both indicate a high rate of mitochondrial fission[77]. DRP1 is upregulated in many cancer types and supports tumor growth and metastasis[78]. Mitochondrial dynamics, including fission, appear to be linked to cell cycle at the G2/M phase, as it is an important checkpoint to ensure an even distribution of mitochondria. Drp1, therefore, plays an important role in mediating cell cycle progression[79]. Interestingly, we found that PRX3 expression in MM cells mediates Drp1 expression and provides evidence that this affects mitochondrial dynamics through activation of Drp1. This was demonstrated in an experiment where cells failed to progress through the G2/M phase when PRX3 expression was inhibited and DRP1 phosphorylation at a key regulatory serine was coincidingly reduced[23]. Disabling the receptor tyrosine kinase MET with MGCD516 in MM cells led to a loss of DRP1 activity that accompanied reduced viability, migration, and invasion[80]. Combining MGCD516 with the proposed mitochondrial fission inhibitor Mdivi-1 induced cell death to a greater extent than either drug used alone[80]. Given that DRP1 expression is upregulated in MM[81], the unique mitochondrial morphologies of MM cells[77] and the connection between mitochondrial redox status, DRP1 activity and cell cycle progression[23] targeting mitochondrial morphology in MM may be a viable therapeutic approach. Lennon *et al.* (2016) also studied the effects of mitochondrial inhibitors metformin and Mdivi-1[77]. Metformin, which inhibits complex I of the electron transport chain, appeared to be highly effective against MM cell lines with a low fractal dimension and a high lacunarity.

Mdivi-1, which inhibits Drp1, was most effective on cell lines with a high lacunarity. This study did not find any correlation with their mitochondrial metabolism measurements. Although the use of treatments like metformin is largely unproven, this suggests that mitochondrial dynamics may be a promising indicator of the effectiveness of cancer treatments[77]. These results suggest that mitochondrial dynamics are a useful target for MM treatment, as well as for other tumors characterized by high mitochondrial oxidant production.

STRATEGIES IN MODULATING MITOCHONDRIA AND REDOX SYSTEMS FOR THERAPEUTIC INTERVENTION

Several studies recognize the difficulty in treating cancer by downregulating ROS production for the same reasons that they are controversial in the role of cancer development. Suppressing ROS with general antioxidants, such as β-carotene and vitamin A, was found to enhance tumor growth[82,83]. Several other studies have criticized the commercial use of antioxidants as a means of cancer prevention. There have been few population-based studies supporting their use[17]. Inducing oxidative stress by selectively increasing cellular ROS or specifically targeting key antioxidant enzymes seems to be a more viable option [Table 1]. One approach is by directly increasing ROS levels within the cell. Many well-known cancer treatments, such as chemotherapy and radiation, already work by inducing oxidative stress[17]. However, caution must be exercised as inducing ROS in cells nonspecifically may induce chemoresistance[84,85]. Additional studies have shown that some ROS-inducing agents may be able to sensitize cancer cells to treatments like radiation therapy. One, in particular, is vitamin C (ascorbate) which acts as a pro-oxidant at higher doses[86] and has been shown to enhance sensitivity to radiation therapy in pancreatic cancer[87,88]. Other studies have shown that depletion of arginine, a critical amino acid in the biosynthesis of proteins, nitric oxide, and polyamine is an actionable approach for therapeutic intervention in argininosuccinate synthase I (ASSI) - negative tumors, including MM[89,90]. Depletion of arginine leads to mitochondrial dysfunction and increased ROS levels[91,92]. Pegargiminase (ADI-PEG 20; ADI) acts to degrade arginine and shows potent activity in ASS1 deficient MM tumors[93]. Given the potent effects on mitochondrial activity, including increased oxygen consumption and ROS levels following arginine depletion, or treatment with ADI-PEG 20, it will be interesting to determine the redox-dependent activity of this approach in the therapeutic response observed[92,94].

A more specific approach to increasing ROS is by targeting antioxidant pathways. As many antioxidant systems are upregulated in cancer cells, they have been identified as important targets for treatment that selectively targets cancer. Thioredoxin (TRX) pathway inhibitors are an important target, as it is suggested that overexpression of TRX leads to chemoresistance to pro-oxidant therapies[59]. For example, *cis*-diamminedichloroplatinum (II)[45] (CDDP, cisplatin) is one of the few chemotherapeutic agents approved for use in MM treatment[95], and its cytotoxicity is partly attributed to its effects on TRX activity. Studies showed that cisplatin cytotoxicity was strongly correlated with thioredoxin reductase (TR) inhibition. This study also showed that an increase in TR expression correlated with cisplatin resistance[96]. Although cisplatin activity is attributed to DNA damage, most of the intracellular platinum content reacts with GSH, forming a bis-(glutathione)-platinum (GS-Pt) complex[97], which, notably, also demonstrated inhibitory effects on TR[98].

Triphenylmethanes, like brilliant green and gentian violet (GV), appear to act by inhibiting the mitochondrial thioredoxin isoform(Trx2)[99]. GV was shown to have potent cytotoxic activity against MM cells in culture and a xenograft model of MM[5]. These studies corroborated initial studies that GV was a potent TRX2 inhibitor as loss of TRX2 expression correlated with cytotoxicity[99]. Additionally, treatment of cells with GV led to significant increases in disulfide-bonded dimers of PRX3, the molecular species reduced

Table 1. Targeting ROS in cancers including malignant mesothelioma

Source citation	Topic	PMID
Chen *et al.*[86] (2007) Alexander *et al.*[87] (2018) Mehdi *et al.*[88] (2021)	Vitamin C (ascorbate)	(17502596) (30254147) (34639220)
Cunniff *et al.*[5] (2015) Nelson *et al.*[6] (2021) Newick *et al.*[55] (2012) Cunniff *et al.*[56] (2013)	Modulating PRX3 and/or FOXM1	(26011724) (33498547) (22761781) (23018647)
Scalcon *et al.*[59] (2018) Zhang *et al.*[99] (2011)	Thioredoxin and thioredoxin reductase inhibitors	(29596885) (21215310)
Omenn *et al.*[82] (1996) Alpha-Tocopherol *et al.*[83] (1994)	Vitamin E and Beta Carotene	(8602180) (8127329)
Gorrini *et al.*[17] (2013) Jezek *et al.*[65] (2021)	Reviews	(24287781) (33418995)

by TRX2 and a significant increase in mitochondrial ROS levels[55].

Studies from our group identified the increased expression of FOXM1 in MM tumors and MM cell lines[55]. This observation led to testing the proposed FOXM1 inhibitor, TS, in preclinical models of MM. TS shows potent anticancer activity in a variety of tumor cell lines[100] and has been proposed to exert its anticancer activity through inhibition of FOXM1[101], the proteasome[102,103] and PRX3 activity[5,6]. Our group has been investigating the molecular mechanism and anticancer activity of TS in preclinical cell and animal models of MM and have deduced that PRX3 is a primary molecular target of TS[5,6,23,55,56,61] [Figure 1]. MM cells are more sensitive to TS compared to normal primary and immortalized mesothelial cell lines, and TS has potent *in vivo* activity in xenografts of human MM cells engrafted to the peritoneal cavity of immunocompromised mice. These studies have collectively shown that TS covalently crosslinks the active site Cys 108 and Cys 229 residues, inducing a stable covalent adduct across the dimer-dimer interface. Crosslinking of PRX3 increases cellular and mitochondrial ROS levels that can be inhibited by pre-treatment with the ROS scavenger N-acetylcysteine (NAC), indicating the redox dependency of TS cytotoxicity. The crosslinking of PRX3 by TS was detectable in tissue resected from mice harboring MM xenografts, providing evidence that the mechanism of PRX3 crosslinking by TS is preserved *in vivo*. Our recent work uncovered the specificity of TS for mitochondrial PRX3 versus the cytosolic peroxiredoxins PRX1 and PRX2. Structural transitions of PRX3, dependent on its oxidation status and the local pH environment of the mitochondrial matrix, support preferential adduction of PRX3 in MM cells. TS treatment of MM cells also leads to a loss in FOXM1 expression. The interplay between TS, PRX3, mROS and FOXM1 remains unclear as knockdown of PRX3 reduces FOXM1 levels and treatment of MM cells with mROS inducing agents (rotenone) leads to loss of FOXM1. Although more research is necessary to dissect this interplay, targeting PRX3 and FOXM1 with TS is an exciting therapeutic approach. TS is currently being tested in the MITOPE phase 1/2 clinical trial to evaluate activity in patients with malignant pleural effusion (MPE) arising from metastatic disease or M (NCT05278975).

CONCLUSIONS

ROS have a complicated role in the development of MM and many other cancers. Although a potent and cancer promoting signaling molecule, increased ROS and adaptations to oxidative stress in cancer cells, including MM, provide a redox vulnerability exploitable through redox-dependent therapies. Several preclinical and established cancer treatments exploit the increased ROS production observed in cancer by directly inducing oxidative stress or targeting complex cellular antioxidant networks. A secondary and complementary approach is targeting mitochondrial dynamics, as they are intertwined with many of the same redox processes. MM cell lines display both increased ROS production and altered mitochondrial

dynamics; therefore, further evaluating these strategies is warranted for the treatment of MM.

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Authors' contributions

Wrote and edited the manuscript: Cote A, Messier T, Cunniff B

Availability of data and materials

Not applicable.

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Conflicts of interest

Brian Cunniff and Terri Messier are paid consultants for RS Oncology, LLC. Brian Cunniff is an equity holder in RS Oncology, LLC.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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