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REVIEWS

Reactive oxygen species: an Achilles' heel of melanoma?

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The successful treatment of melanoma has been hampered by the unique biology of this cancer. Fortunately, research to further our understanding of how melanoma cells differ from normal tissues has led to the discovery of potential new avenues of attack. One promising strategy relates to targeting the excess free radicals produced by melanomas. Melanocyte transformation into cancer is associated with significant structural alterations in the melanosome. In addition to pigment production, melanosomes also protect the cell by scavenging free radicals generated by sunlight and cellular metabolism. In melanoma, the disrupted and disorganized melanosome structure reverses this process. Melanosomes found in melanoma produce free radicals, such as hydrogen peroxide, furthering DNA damage. Melanosome generation of reactive oxygen species (ROS), in tandem with those generated by cancer metabolism, activate cellular signal transduction pathways that prevent cell death. ROS activation of proto-oncogene pathways in melanoma contributes to their resistance to chemotherapy. Fortunately, it may be possible to target these free radicals, just as Paris was able to successfully target Achilles' heel. The use of agents that block ROS scavenging, such as ATN-224 and disulfiram, have been explored clinically. A recent randomized Phase II trial with elesclomol, an agent that generates ROS, in combination with paclitaxel led to improved patient survival, suggesting that this may be a viable approach to advance the treatment of melanoma.

KEYWORDS: arsenic trioxide • ATN-224 • buthionine sulfoximine • disulfiram • elesclomol • glutathione • melanoma • MGD • motexafin gadolinium • reactive oxygen species

Melanoma: a recalcitrant skin cancer

Malignant melanoma is one of the most lethal solid tumors. In the USA, melanoma is the sixth most common cancer in men and the seventh in women. New melanomas will be diagnosed in 62,480 patients in 2008, while 8420 will die [1]. When diagnosed early, melanoma is curable by surgery alone, with 80% of patients relapse free 10 years after surgery. When disease has spread to distant lymph nodes or metastasized (stage IV) it becomes refractory to common therapies and, therefore, incurable [2].

Dacarbazine (DTIC) and its oral analog, temozolomide, are currently considered to be standard first-line treatments for metastatic disease, with response rates of approximately 10–15% [3–5]. The efficacy of these agents is disappointing, however, with no improvement in median overall survival (OS) [5]. The increasing incidence of melanoma and poor survival for patients with metastatic disease has fostered the development of novel approaches to the treatment of this disease. Over the past two decades, a better appreciation of signaling pathways in cancer has advanced our

understanding of melanocyte and melanoma biology, opening new doors to the development of targeted therapy [6–8].

Melanin biology & reactive oxygen species

One of the unique hallmarks of melanoma biology is the presence of melanosomes, pigment containing organelles that contain melanin synthesis apparatus. While normal melanosomes function to scavenge reactive oxygen species (ROS), melanoma cells contain structurally aberrant melanosomes that generate free radicals (FIGURE 1) [8–10]. Melanin generation of radical species is thought to be related, in part, to an increase in transition metal binding to the altered pigment structure [9–14]. Chronic production of oxidative stress, in combination with ultraviolet (UV)A and UVB radiation exposure, may contribute to the transformation process of dysplastic nevi, leading to frank malignancy and an intrinsically drug-resistant phenotype [10,14–16]. On the other hand, the relatively melanoma-specific accumulation of ROS may provide a unique therapeutic target [16,17].

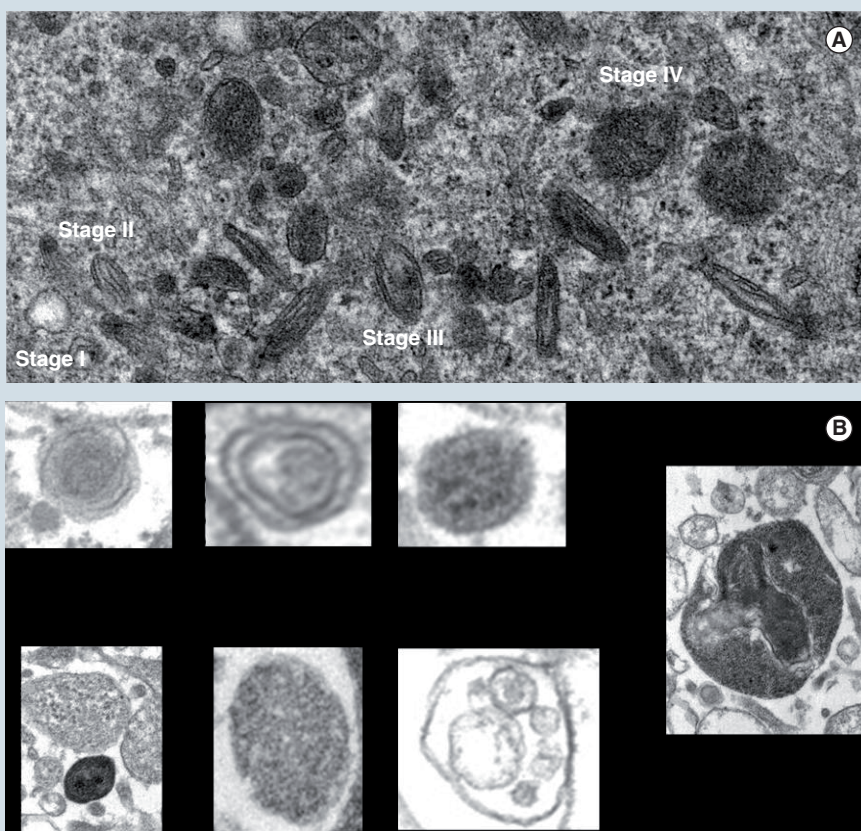


Figure 1. Aberrant melanosome structure in melanoma compared with normal melanocytes. (A) Melanosomes in normal melanocytes. **(B)** Melanosomes in melanoma.

ROS in cell signaling

Reactive oxygen species are emerging as critically important signaling molecules involved in cellular proliferation, apoptosis suppression and angiogenesis in melanoma [16–21]. In addition to *PTEN* and *BRAF* mutations, proto-oncogene pathway activation can occur in melanoma as a result of ROS potentiation of *AKT* signaling by inhibition of *PTEN* phosphatase activity (FIGURE 2). The tumor-suppressor gene product, p53, is also redox sensitive and is stabilized under hypoxic conditions [20]. Functions of p53 include cell cycle arrest in the presence of DNA damage via upregulation of p21, induction of apoptosis via upregulation of BAX, and suppression of angiogenesis via upregulation of thrombospondin-1, an endogenous inhibitor of new vessel formation. Whereas direct mutation of p53 is infrequent in sporadic melanoma, loss of function of p53 is rather common [6]. p53 is regulated, in part, by its association with MDM2, which promotes p53 degradation. In familial melanoma, significant percentages have deletions or point mutations in cyclin dependent kinase inhibitor (CDKN)2A, which codes for p14 and p16. p14 normally functions to stabilize p53 by blocking its interaction with MDM2, while p16 normally acts to suppress retinoblastoma (RB) protein phosphorylation, slowing cell cycle progression. *AKT/PTEN* pathways enhance cell cycle progression and work in conjunction with the loss of function of p16 and p53 tumor suppressor, increasing tumor growth rates and blocking intrinsic apoptosis pathways [6,7].

Reactive oxygen species generated by hypoxic mitochondria and NADPH oxidases augment these growth pathways by stabilizing hypoxia inducible factor (HIF) 1- α , blocking *PTEN*-mediated inhibition of *AKT* signaling and activating *NF- κ B* nuclear localization [16]. Various growth factor receptors on the melanoma cell surface signal through *AKT* to increase levels of *BCL2* family proteins that can block apoptosis. *NF- κ B* mediates transactivation of genes that are also related to suppression of apoptosis and angiogenesis [23–28]. Thus, chronic oxidative stress associated with abnormal melanosomal structure and increased levels of transition metals results in a physiologic state favoring cell proliferation and resistance to conventional chemotherapy [6,9,15,26,29]. Knowledge of these pathways has generated a great deal of interest in selective drug targeting to reverse these processes [30–33]. Unfortunately, no single targeted therapy has emerged that improves OS for unselected patients. To date, trials evaluating DTIC in combination with knockdown of *BCL2* using the antisense agent oblimersen, sorafenib-mediated inhibition of *BRAF* in combination with DTIC and the *MTOR* inhibitor, CCI779, as a single agent have all yielded negative results overall with respect to a survival advantage and, while some subsets may benefit, this

has yet to be further validated [34–36]. In fact, an OS benefit from a systemic approach has yet to be demonstrated in a randomized clinical trial of metastatic melanoma. The lack of success for these agents is probably related to the redundancy of death-suppression pathways and interpatient heterogeneity of pathway activation [37]. These studies highlight the potential need for combining agents in a patient tumor biology-specific fashion [29,32].

ROS as a therapeutic target

Elevated ROS levels in melanoma may, in part, result from abnormal melanosomal structure, leading to abnormal proliferation and apoptosis suppression [9–11]. It would be of interest if this relatively melanoma-specific defect could be targeted therapeutically. One approach would be to increase ROS scavenging, thereby decreasing ROS levels and disrupting growth signaling. Agents known to provide increased scavenging include *N*-acetylcysteine (NAC) and amifostine. However, only limited data suggest that this may be of benefit, and concern that antioxidants may protect cancer cells from the effects of conventional therapy has dampened enthusiasm for this approach [38]. An alternative approach that is gaining some traction is to take advantage of the toxic nature of the ROS formed in melanoma cells [16]. Depletion of ROS scavengers, such as glutathione (GSH), can allow free radicals to accumulate to

extreme levels, thereby inducing apoptosis. In this regard, depletion of ROS scavengers could be therapeutic. Excessive levels of ROS induce apoptosis via activation of DNA damage-repair pathways and by modulating mitochondrial pore opening [16,40,41]. Agents capable of blocking ROS scavenging include L-buthionine-S,R-sulfoximine (BSO), which inhibits GSH synthesis, ATN-224, which inhibits Cu,Zn-superoxide dismutase (SOD)1, arsenic trioxide, which depletes GSH, and disulfiram (DSF), which interferes with GSH recycling by competing with oxidized GSH for the active site of GSH reductase [42–74]. Agents that increase ROS levels include elesclomol and motexafin gadolinium (MGd) (FIGURE 3) [75–82].

Preclinical and clinical data indicate that GSH depletion by BSO is selectively toxic against melanoma [39,44,45]. Melanoma tumor specimens are more sensitive to GSH depletion than breast or ovarian tumors [39]. Treatment of mice bearing melanoma with BSO increased survival and inhibited formation of metastatic disease [46]. Melanoma patients on Phase I studies of infusional BSO given in combination with melphalan responded to this therapy [47,48]. These early findings supported the notion that manipulation of ROS scavengers may be a fruitful approach.

Similarly, inhibition of SOD1 by ATN-224 demonstrated modest antimelanoma activity *in vitro* and *in vivo* [49,50]. ATN-224 is a second-generation tetrathiomolybdate analog and is thought to inhibit copper- and zinc-dependent SOD1 through copper chelation. *In vitro*, ATN-224 promotes apoptosis and inhibits proliferation in both cancer and endothelial cells [51,52]. It has been suggested that redox enzyme SOD1 is a therapeutic target for angiogenesis in addition to tumor growth [53]. ATN-224 has undergone testing in Phase I trials [54,55] and is undergoing testing in Phase II clinical trials for melanoma.

Disulfiram is a member of the dithiocarbamate family. Dithiocarbamates are associated with metal chelation and intracellular oxidative stress. DSF has been shown to be both zinc and copper dependent [56–59] and is selectively toxic to melanoma cells [60]. It increases superoxide levels while decreasing hydrogen peroxide levels, thereby pointing to SOD as its likely target. DSF causes apoptosis in melanoma with an effect comparable to BSO. *N*-acetyl-L-cysteine (NAC), a glutathione precursor and antioxidant, reverses DSF-induced cell death [61,62]. Similar to ATN-224, DSF has also been shown to inhibit angiogenesis [58]. DSF has been found to be effective at inhibiting tumor growth in xenograft models [59] and has moderate toxicity in patients. Anecdotally, DSF in combination with zinc caused considerable regression of liver metastases in a patient with uveal melanoma [63]. DSF is currently being investigated in combination with arsenic trioxide (As_2O_3) for patients with metastatic melanoma.

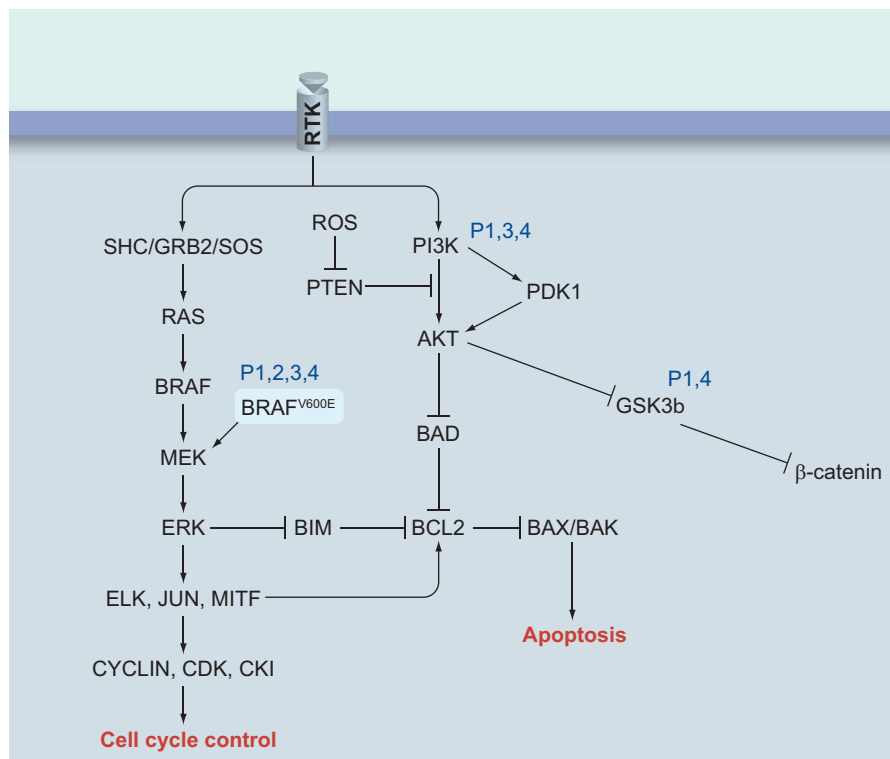


Figure 2. Cell-signaling pathways in melanoma.

Arsenic trioxide is an agent commonly used in the clinic for the treatment of acute promyelocytic leukemia (AML). Similar to BSO, ATN-224 and DSF, it has been shown to promote apoptosis through an ROS-dependent pathway [64]. GSH content is inversely proportional to sensitivity of cell lines to As_2O_3 , and cells with increased ROS levels are more sensitive to As_2O_3 [65,66]. GSH depletion by BSO in combination with As_2O_3 has been shown to overcome As_2O_3 resistance in leukemia and solid tumors [67,68]. Results of Phase I trials in solid tumors and Phase II trials in melanoma of single-agent As_2O_3 or As_2O_3 in combination with temozolomide reported little or no activity [69–71]. These data support the rationale for combining As_2O_3 with DSF, other inhibitors that block ROS scavenging, or agents that induce ROS.

Motexafin gadolinium is a redox-active agent that has been shown to increase ROS *in vitro*. MGd is an aromatic macrocyclic compound with a strong affinity for electrons and is more easily reduced than molecular oxygen. Reduction of MGd under neutral aqueous conditions leads to superoxide formation, which is greatly enhanced in the presence of ascorbate [75]. MGd has been shown *in vitro* and *in vivo* to localize to tumor cells [76–78]. Apoptosis is promoted by MGd in a caspase and mitochondrial-dependent manner. Increased production of intracellular ROS in cancer cells treated with MGd has been demonstrated *in vitro*. MGd increases ROS in cancer cell lines in a dose-dependent manner and was also found to reduce GSH levels in chemotherapy sensitive, but not chemotherapy resistant cell lines [79].

Elesclomol was originally identified through *in vitro* screening of agents that would enhance the cytotoxicity of paclitaxel. Elesclomol also potentiates the activity of other cytotoxic

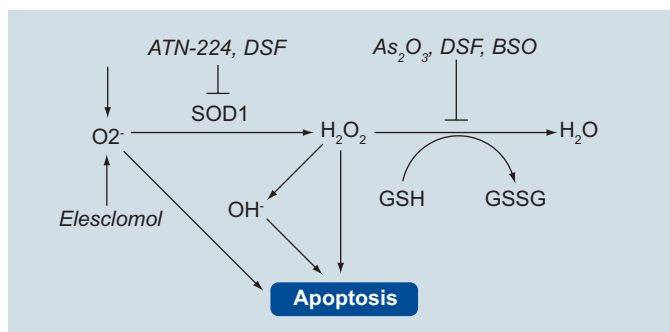


Figure 3. Sites of action of reactive oxygen species-modulating agents.

agents such as rituximab, gemcitabine and docetaxel. As with MGd, elesclomol is believed to sensitize cancer cells through induction of ROS. Induction of ROS was shown *in vitro*, and reversal of ROS induction was achieved through pretreatment with antioxidant NAC. After 6 h of treatment with elesclomol, melanoma cells upregulated transcript levels of genes indicative of cells undergoing oxidative stress, such as genes regulated by heat shock stress response and metallothionein genes. Treatment with NAC also reversed this transcriptional response [80]. Elesclomol was active as a single agent at high doses in an M14 human melanoma xenograft model, while low doses of elesclomol were effective in combination with paclitaxel without increasing toxicity. A recent Phase II trial randomizing patients with metastatic melanoma to paclitaxel alone or paclitaxel in combination with elesclomol demonstrated a significant improvement (11.9 vs 7.8 months) in median OS for patients who received elesclomol. A Phase III trial has recently begun that will examine this comparison in 630 patients to determine if the combination of paclitaxel and elesclomol will emerge as a new standard treatment for melanoma [80–82].

As with all agents, off target toxicity has been seen with ROS targeting agents. In the Phase II trial of paclitaxel versus elesclomol in combination with paclitaxel, the addition of elesclomol carried little additional toxicity. Of the adverse events in either group, toxicity due to paclitaxel alone was more common than toxicity due to the combination, with the exception of neutropenia which occurred in 6% of patients randomized to the combination and no patients randomized to paclitaxel alone [80]. In the Phase III trial of MGd, grade 3 toxicities were found to be cardiovascular, hepatic and neurologic [83]. DSF also carries hepatic and neurologic toxicity, whereas arsenic trioxide toxicity is hematologic, neurologic, endocrine and cardiovascular.

In addition to off-target toxicity, these ROS-modulating agents may have off-target efficacy. It has been observed in our laboratory that cells resistant to BSO divide more slowly than those sensitive to BSO. It is likely that, in addition to targeting the Achilles' heel of melanoma, BSO and other ROS-modulating agents can act on other targets in rapidly proliferating cells, potentially enhancing the toxicity of chemotherapy [43]. Many of these agents have multiple targets and promiscuous activity. The potential off-target efficacy, as well as toxicity,

must be taken into account in the design of new clinical trials. It is important to keep an open mind as different or unexpected off-target effects may be associated with this class of agents.

Melanin content as a predictor of response to excess ROS levels

Individualization of therapy requires biomarkers predictive of response. Currently, melanin content appears to be predictive of response to agents that block ROS scavenging *in vitro* [38,39]. Further work in this area will be needed to optimize treatment selection with these agents.

Five-year view

Studies in cancer biology have recently revealed the role of ROS in cell signaling and the redundancy of death-suppressor pathways in melanoma associated with cancer progression and drug resistance. However, there may be particular points of attack within an individual patient's tumor biology that can be exploited. To take advantage of this potential, advances in methods to characterize patient tumor specimens are needed. Gene array profiling has been complicated by the excessive amounts of information generated. With advances in bioinformatics over the next few years, it may become possible to sample a patient's tumor tissue, extract RNA and identify vulnerable links that can be targeted. This approach will depend on the expansion of our repertoire of targeted therapies that can be brought to bear against the broad spectrum of pathway variation expressed between patients. It is likely that, in the near future, most targeted therapies will be given in combination with conventional therapies to trigger tumor cell apoptosis. The strategic tailoring of cytotoxics, ROS-modulating agents and antiangiogenic therapies may emerge as the viable strategy while we await the development of chemoprevention agents that will arrest the development of melanoma in its infancy.

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Key issues

- Melanoma is relatively unique among tumors in its generation of excessive levels of reactive oxygen species (ROS).
- ROS modulate various cellular signaling pathways, promoting proliferation, drug resistance and angiogenesis.
- Utilization of agents that block the scavenging of ROS or that increase levels of intracellular ROS is an emerging area of therapeutic development.
- Biomarkers linked to ROS dysregulation are needed to optimize treatment selection.

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