

## Drug resistance in melanoma: Mechanisms, apoptosis, and new potential therapeutic targets

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### Abstract

Melanoma is the most aggressive form of skin cancer. Patients with advanced disease, such as lymph node involvement and distant metastases, have 5-year survival rates of 50% and 10–20%, respectively. This poor prognosis largely results from resistance to conventional chemotherapy, namely cytotoxic drugs. The basis for drug resistance in melanoma is most likely dysregulation of apoptosis, although other mechanisms including drug transport, detoxification, and enhanced DNA repair may also play a role. Defects at multiple levels and in both major apoptotic pathways have been described in melanoma. Our laboratory has identified an inhibitor of apoptosis, termed survivin, that is expressed in melanoma and required for maintenance of melanoma cell viability. Targeting of survivin and other apoptotic regulators increases the sensitivity of melanoma cells to cytotoxic drugs, and may provide a promising new therapeutic approach to cancer.

### Melanoma: prognosis and treatment

The incidence of melanoma has doubled over the past few decades and is increasing more rapidly than any other cancer [1]; it accounts for over 7000 deaths per year in the US [2]. When treated early, however, melanoma is often curable by surgical excision, as patients with minimally invasive lesions have a 10-year survival greater than 95% [3]. By contrast, long-term survival decreases to 50% once tumor has spread to lymph nodes and is only 10–20% for patients with extracutaneous metastases [4]. Patients with advanced disease are generally refractory to conventional treatments, accounting for their poor prognosis. Although new therapies have become available, unfortunately none have proved better than traditional chemotherapy in a majority of patients [5].

### Drug resistance in melanoma

The alkylating agent dacarbazine (DTIC) was the first chemotherapeutic drug used for advanced melanoma,

and remains the only one approved by the FDA for this purpose. Overall response rates range from 10% to 20%, with complete remissions in only 5% of patients [6]. Other chemotherapeutic drugs have been tried, including nitrosoureas (carmustine, lomustine), vinca alkaloids (vincristine, vinblastine), platinum compounds (cisplatin, carboplatin) and taxanes (Taxol, docetaxel), but none of these single agents represented an improvement over DTIC [7]. Although various combination chemotherapies did increase response rates in some studies, none succeeded in prolonging survival [8].

Experiments using melanoma xenografts (in immunodeficient mice) derived from patient tumors have confirmed the resistance of melanoma cells to DTIC, cisplatin, and other chemotherapeutic drugs *in vivo* [9]. Numerous assay systems have been developed to assess chemosensitivity *in vitro*, and most involve incubation of melanoma cells with a particular drug for a brief period followed by seeding in soft agar [10]. Schadendorf et al. [11] used this approach to predict chemoresistance *in vivo* for 19 melanoma tumor samples.

### Potential mechanisms of drug resistance

The mechanisms conferring this intrinsic drug resistance in melanoma cells are poorly understood. Grottko et al. [12] found 11 genes that were differentially expressed upon acquisition of etoposide resistance in melanoma cells, but most were of unknown function and remain to be characterized. Multiple mechanisms identified in chemoresistance of other tumor cell types have been investigated in melanoma.

#### *Drug transport*

The two classes of ATP-dependent drug transporter proteins, P-glycoprotein (Pgp) and the multidrug resistance-associated proteins (MRPs), mediate drug efflux that reduces drug accumulation and renders tumor cells resistant to the cytotoxic effects of many anticancer agents. Most melanoma cell lines do not express Pgp [13], and its expression is not induced upon exposure to chemotherapeutic drugs [13,14]. On the other hand, MRP is expressed in melanoma [15,16], but in one study [15] its expression did not increase after chemotherapy. Thus, induction of drug transporters does not appear to be a major factor in mediating drug resistance in melanoma, although not all drug transporters have been examined.

#### *Detoxification by glutathione conjugation*

The enzyme glutathione-S-transferase is involved in the detoxification of many drugs (particularly alkylating agents) by conjugation to glutathione. Glutathione transferase levels were found to be higher in melanoma lesions compared to benign melanocytic nevi [17,18]. However, glutathione and glutathione transferase levels varied greatly within a panel of melanomas and did not correlate with course of tumor progression, chemotherapy treatment, or clinical response [19]. Moreover, glutathione depletion was not found to increase cytotoxicity of cisplatin or affect drug levels in melanoma cells [20].

#### *Topoisomerases*

Topoisomerase (Topo), the target of inhibitory cytotoxic agents such as etoposide, is a key enzyme involved in DNA replication and transcription and may be mutated or repressed in some cancers. Expression of both Topo I [21] and Topo II [22] has been found

in series of primary and metastatic melanomas. Etoposide resistance in melanoma cells has been associated both with decreased expression [23] and mutation or deletion [24] of Topo II. In a study of choroidal melanoma, however, expression of topoisomerase II did not reliably predict sensitivity or resistance [25].

#### *DNA repair*

Alkylating agents form adducts at the O<sup>6</sup> position of guanine, resulting in interstrand DNA crosslinks that are cytotoxic. Repair of these adducts by the DNA repair enzyme O<sup>6</sup>-alkylguanine DNA alkyltransferase (AGT) thus impairs the cytotoxic action of alkylating agents and mediates a major resistance pathway for these drugs. Drug-resistant melanoma cell lines exhibit increased (base excision) repair of DNA damage [26]. One study [27] found increased but variable AGT activity in melanoma, higher in metastatic lesions than in primary tumors, and higher in melanoma metastases after DTIC treatment, while another analysis of metastatic lesions [28] found AGT mRNA absent in over a third. The increased expression of AGT in fotemustine-resistant melanoma cells was shown to be associated with intragenic hypermethylation [29]. In a clinical trial of temozolomide in advanced melanoma, however, pretreatment levels of AGT were not predictive of clinical response [30].

#### *Ras mutations*

Activating ras mutations are found in approximately 15% of melanomas, leading to increased growth potential [31]. N-ras mutations in particular are associated with chemoresistance [32]. The underlying mechanism for ras-mediated drug resistance is the resistance to apoptosis, likely due to increased levels of Bcl-2 [33] (see below).

#### *Dysregulation of apoptosis*

Most chemotherapeutic drugs ultimately act through induction of apoptosis (programmed cell death) [34], and their reduced efficacy in melanoma likely relates to a relative inability to induce apoptosis in melanoma cells compared with other malignant cell types [35]. Indeed, cells within melanoma lesions demonstrate an inherently low level of spontaneous apoptosis [36,37], and resistance to apoptosis has been correlated with increased metastatic potential in animal models of

melanoma [38]. There is also evidence for increased resistance to apoptosis in melanocytic nevi compared to isolated melanocytes [39], suggesting that the acquisition of apoptosis resistance may be an early step in the malignant transformation from normal melanocyte to melanoma.

### Molecular basis for apoptosis resistance in melanoma

On a molecular level, apoptosis represents a built-in genetic program consisting of several pathways that are summarized in Figure 1.

#### Overview of pathways and regulators of apoptosis

The 'extrinsic' apoptotic pathway is triggered by binding of Fas ligand, tumor necrosis factor (TNF), or TNF-related cytokines (i.e. TRAIL) to extracellular membrane receptors (death receptors) that lead to activation of caspase-8 [40]. Caspases (cysteine proteases

that cleave after aspartic acid) are constitutive proenzymes that are activated upon cleavage by other caspases, resulting in a proteolytic cascade (Figure 1) [41]. Death receptor signaling can also activate an alternate 'intrinsic' pathway, involving mitochondrial release of cytochrome *c*, which in combination with the adapter molecule Apaf-1 leads to activation of caspase-9 (Figure 1) [42,43]. These two pathways converge with the activation of terminal caspase-3 and -7 that cleave proteins involved in nuclear membrane and cytoskeletal structure, DNA repair, and replication systems [44].

Apoptosis is controlled, in all cells, by a cast of regulatory molecules that maintain the balance between cell survival and cell death at multiple levels of each pathway. For example, the external pathway is regulated in part by coordinated expression of death-inducing and decoy receptors on the cell surface [40]. In addition, a structural homologue of caspase-8 (FLIP) has been described [45] that lacks proteolytic activity and blocks apoptotic signaling through death receptors. The intrinsic pathway is primarily controlled by Bcl-2 family proteins, which comprise molecules with pro- and anti-apoptotic activity regulating mitochondrial homeostasis and particularly cytochrome *c* release

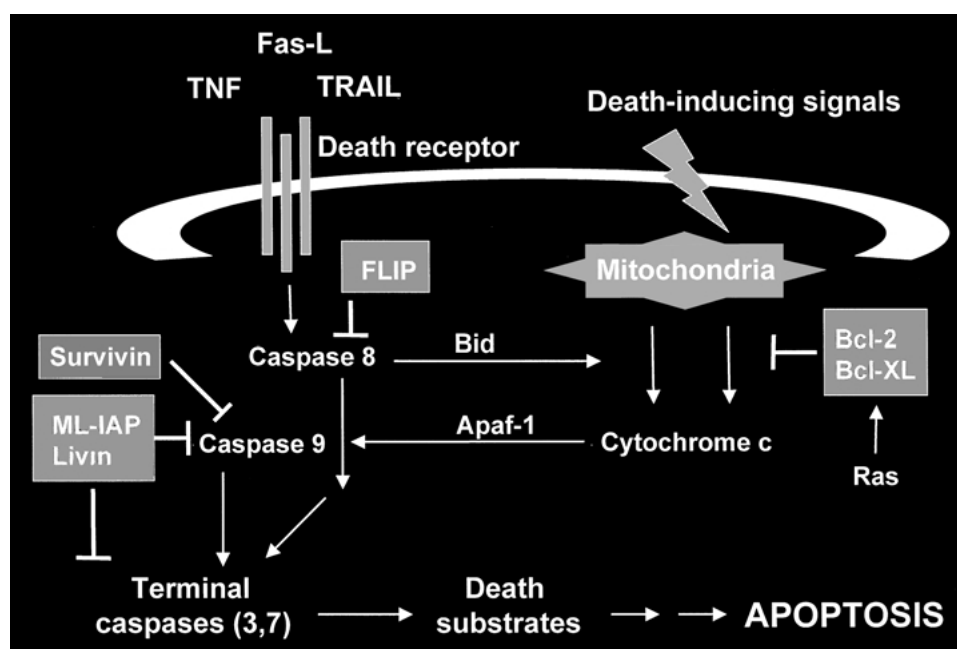


Figure 1. Schematic of apoptotic pathways and regulators. The predominant pathways leading to apoptosis involve either death receptor signaling or mitochondrial release of cytochrome *c*. FLIP, survivin and ML-IAP/Livin act by antagonizing caspases. Bcl-2 and Bcl-X<sub>L</sub> block cytochrome *c* release.

(Figure 1) [46]. A functional cross-talk and amplification loop between intrinsic and extrinsic apoptotic pathways appears to involve caspase-8-dependent cleavage of a pro-apoptotic Bcl-2 family member, Bid, which then promotes cytochrome *c* release (Figure 1) [47].

The inhibitor of apoptosis (IAP) proteins (NAIP, cIAP-1, cIAP-2, XIAP, survivin, and Livin/ML-IAP) comprise an additional gene family of apoptotic regulators that are characterized structurally by the presence of one or more conserved zinc-binding domains (termed baculovirus IAP repeat, BIR) [48]. IAP proteins protect against a variety of apoptotic stimuli including TNF, Fas ligand, UV radiation, cytochrome *c*, caspases, viral infection, growth factor withdrawal, and various chemotherapeutic drugs [49]. They function as caspase inhibitors, exerting their anti-apoptotic activities at both upstream [50–52] and downstream [53–55] sites in the caspase cascade. The IAPs are held in check by physical interaction with the mitochondrial protein Smac/DIABLO, which relieves the IAP-caspase interaction, thus freeing the caspase for downstream completion of apoptosis [56,57].

Survivin is an IAP protein with a single BIR domain [58], and differs from other IAP proteins and apoptotic regulators in several respects. Survivin expression is ubiquitous during fetal development [59] but undetectable or found at very low level in terminally differentiated adult tissues [58]. It is expressed in most transformed cell lines [55], and a recent analysis of human ‘transcriptomes’ found it to be one of only 40 genes expressed in cancers but not normal tissues [60]. On a molecular level, survivin is upregulated 40-fold in the G<sub>2</sub>/M phase of the cell cycle and physically associates with the mitotic spindle in dividing cells [61]. Interference with its expression or function in malignant cells triggers apoptosis in G<sub>2</sub>/M, and a cell-division defect characterized by aberrant mitotic figures and multinucleated polyploid cells [62]. Recent work from our laboratory demonstrated that survivin averts apoptosis at the level of caspase-9 [52] rather than the terminal caspase-3, as suggested by others [63].

#### *Apoptotic regulators in melanoma*

Most of the known apoptotic regulators can be found in melanoma (see Figure 1), but in some cases there is preferential expression of apoptotic inhibitors that may account for increased apoptosis resistance in melanoma cells compared to normal melanocytic or other cell

types. Alternatively, impairment of a pro-apoptotic pathway or loss/inactivation of a pro-apoptotic factor may lead to apoptosis resistance.

Several groups [64–66] have reported defective death receptor signaling in melanoma cells. Receptor levels may be downregulated in TRAIL-resistant cells [67,68], although this appears not to be the case in Fas-resistant cells [66] which were found defective in cytochrome *c* release [69]. Irmeler et al. [70] reported high levels in melanoma of the apoptosis inhibitor FLIP, an important negative regulator of death receptor signaling.

With respect to the Bcl-2 family, several groups [71–73] found Bcl-2 expression in melanoma lesions comparable to that in nevi, whereas another [74] found decreased Bcl-2 expression in melanoma. In a large series of uveal melanoma, however, levels of Bcl-2 expression did not correlate with disease progression [75]. With respect to other Bcl-2 family members, Tang et al. [74] reported over-expression of both the pro-apoptotic protein Bax and the anti-apoptotic proteins Bcl-X<sub>L</sub> and Mcl-1 in melanoma lesions compared to nevi. On the other hand, Selzer et al. [73] found expression of Bax, Bad, Bak, and Bcl-X<sub>L</sub> in melanoma cell lines to be comparable to that in normal melanocytes.

The tumor suppressor p53 promotes cell cycle arrest and apoptosis in response to DNA damage, and its mutation or deletion in cancer cells has been associated with chemoresistance [76]. Although p53 mutations are rare in melanoma [77], Soengas et al. [78] have shown that Apaf-1, a critical downstream mediator of p53-dependent apoptosis [79], is deleted and inactivated by methylation in metastatic melanomas.

Our laboratory has investigated the expression and function of survivin in both melanoma lesions and cell lines [80]. We found survivin expression in all metastatic lesions and cell lines, and the majority (>85%) of invasive melanomas, but not in normal melanocytes. Interestingly, survivin was also found in melanocytic nevi, suggesting that its expression occurs very early in melanoma progression. Survivin is a survival factor for melanoma cells, as blocking its expression triggers spontaneous apoptosis.

In addition to survivin, another IAP molecule with a single BIR domain has been identified in melanoma cells by two groups, and given the names ML-IAP [81] and Livin [82]. ML-IAP/Livin is absent in most adult tissues including melanocytes, and expressed in developmental tissues and cancer cell lines, with high expression in melanoma lines. Physical interactions of ML-IAP/Livin with both upstream (caspase-9) and

downstream caspases (caspase-3 and -7) have been demonstrated. Like survivin, ML-IAP/Livin also associates with the cytoskeleton and appears required for melanoma cell viability as antisense treatment induced apoptosis in a melanoma line [82].

### New potential therapeutic targets

Correcting the dysfunctional apoptotic program in melanoma cells may be a key requisite to overcome drug resistance and improve clinical outcome. Characterization of the apoptotic regulators in melanoma has suggested multiple potential strategies for either directly inducing apoptosis in melanoma cells and/or increasing their susceptibility to cytotoxic drug therapy.

#### *Blocking apoptotic inhibitors*

Targeting survivin and ML-IAP/Livin is particularly attractive given their relatively selective expression in tumor cells and their function as cell survival factors. As noted above, both molecules have been successfully targeted using antisense. In the case of survivin, we have shown that mutation of the conserved Cys<sup>84</sup> in the BIR domain results in a potent dominant-negative mutant that blocks endogenous survivin function [80]. Recently, we have characterized a phosphorylation-defective Thr<sup>34</sup> BIR mutant that prevents tumor formation and slows the growth of established tumors in a melanoma xenograft model [83]. This particular mutant was also capable of enhancing the sensitivity of melanoma cells to cisplatin *in vitro* [83]. A small survivin antisense oligonucleotide has also been shown to induce apoptosis and increase etoposide sensitivity in adenocarcinoma cells [84].

Although expressed in normal as well as malignant cells, Bcl-2 also appears to be a promising target for melanoma therapy. Rieber et al. [85] demonstrated that a Bcl-2/Bcl-XL bispecific antisense oligonucleotide induces p53-independent apoptosis in human melanoma cells. Jansen et al. [86] found that Bcl-2 antisense chemosensitizes xenografted human melanoma cells to DTIC. In a phase I–II clinical study of patients with advanced melanoma (expressing Bcl-2) who received Bcl-2 antisense and DTIC, 40% exhibited decreased Bcl-2 expression, tumor cell apoptosis, and antitumor responses [87].

Given the role of activated Ras as an upstream regulator of Bcl-2, Jansen et al. have investigated the

effects of targeting Ras in melanoma. They reported that *S-trans, trans*-farnesylthiosalicylic acid, a recently discovered Ras antagonist, blocks melanoma growth both *in vitro* and *in vivo* [88]. Similarly, Ras antisense decreases melanoma growth both *in vitro* and *in vivo* [89].

#### *Death receptor signaling*

TRAIL also offers the advantage of inducing apoptosis in most melanoma cell lines but not normal tissues [67]. Griffith et al. [90] have demonstrated increased apoptosis in melanoma cells infected with a TRAIL-expressing adenovirus. In addition, dominant-negative mutants [91] and soluble inhibitors [92] of NF- $\kappa$ B have been used to increase the sensitivity of melanoma cells to death receptor signaling.

#### *Reestablishing the p53 pathway*

Cirielli et al. [93] have shown that adenoviral-mediated gene transfer of wild-type p53 increased apoptosis and inhibited melanoma cell growth both *in vitro* and *in vivo*. Retroviral transduction of the human type I consensus interferon coding sequence into melanoma cell lines was associated with upregulation of p53, downregulation of Bcl-2, and increased sensitivity to cisplatin [94]. Cisplatin administration to nude mice bearing interferon-producing tumors resulted in complete tumor regression, while only a partial tumor inhibition was observed upon cisplatin treatment of mice bearing parental tumors [94]. Finally, Soengas et al. [78] were able to restore sensitivity to adriamycin in melanoma cells by reactivating (demethylating) Apaf-1 with 5-aza-2'-deoxycytidine.

### Summary

Of many potential mechanisms, it is likely that dysregulation of apoptosis constitutes one of the key factors responsible for acquired drug resistance in melanoma. Identifying the molecular aspects of apoptosis resistance in melanoma offers new potential therapeutic targets that alone, or in combination with conventional cytotoxic drugs, may improve prognosis for melanoma patients with advanced disease.

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