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On the TRAIL to Overcome BRAF-Inhibitor Resistance

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BRAF inhibition has been an instant, although short-lasting, success in BRAF-mutated melanoma treatment. Novel data by Berger *et al.* now suggest that BRAF-inhibitor-mediated "priming to death" facilitates tumor necrosis factor–related apoptosis-inducing ligand–mediated apoptosis. We give an overview about the importance of the crosstalk of extrinsic and mitochondrial apoptotic signaling and propose other combination therapies that may prevent or overcome secondary resistance in melanoma.

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In comparison with previous decades, melanoma therapy has made tremendous progress in the past 10 years. This breathtaking change began in 2001 with the identification of BRAF V600 mutations in about half of all melanomas. Subsequently, the successful development of small molecules able to block signaling by mutated BRAF resulted in clinical studies that showed clinical benefit unequivocally in melanoma patients carrying BRAF mutations (Chapman *et al.*, 2011). In addition to the BRAF inhibitors vemurafenib and later dabrafenib, the MEK (MAPK/ERK kinase) inhibitor trametinib has now been approved for use by the Federal Drug Administration. Indicating the substantial change in clinical perspective in the first decade of the 21st century, objective tumor responses have become clinical realities, rather than exceptions for patients with metastatic melanoma. However, most patients relapse within months, suggesting that secondary resistance precludes long-term survival. Therefore, selection of resistant melanoma cells during drug treatment, based on the activation of alternative signaling pathways, is evident and it makes understanding and overcoming secondary resistance with combination therapies a major focus of dermatooncologists. Currently, combination therapies target both BRAF and overactive MEK (Flaherty *et al.*, 2012), but other combination therapies are also envisioned (Uzdensky *et al.*, 2013). One important hallmark of cancer that holds promise for overcoming primary or secondary resistance to treatment is the breaking of cell death resistance (Hanahan and Weinberg, 2011). Resistance to RAF inhibitors is thought to be the result of intratumoral ''selection'', and this favors concepts of (a) tumor cell plasticity beyond genetic changes and (b) deregulation of metabolic programs upon BRAF-inhibitor treatment (Haq *et al.*, 2013; Knight *et al.*, 2013). To identify additional targets that may lead to improved combination therapies, a detailed knowledge of signaling events in BRAF-inhibitor-treated RAF-mutated melanomas is important. For example, counter-regulation of the apoptotic process by the upregulation of antiapoptotic proteins and deregulation of intracellular proliferative signaling cascades by the permanent activation of RAS/RAF/MEK/ERK or PI3K/PTEN/AKT is well recognized, and it may contribute to secondary resistance (Wolchok *et al.*, 2013).

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Combined therapies that first (or concomitantly) inhibit different oncogenic pathways or are followed by the activation of the apoptotic signaling machinery are attractive therapeutic strategies for metastatic melanoma. Such therapies may prevent—or delay—the development of secondary resistance, with clinical benefit. One such signaling pathway is the extrinsic or death receptor (DR)-induced cell death pathway. Extrinsic cell death is activated by extracellular stimuli such as TRAIL (tumor necrosis factor–related apoptosis-inducing ligand), CD95L, or tumor necrosis factor (also called death ligands). Stimulation of these receptors results in the initiation of an intracellular signaling cascade that triggers apoptosis (Figure 1). The most critical step during this process is the autoproteolytic activation of procaspase-8 within the death-inducing signaling complex (DISC) that also contains the adapter molecule FADD and the receptor-interacting protein 1 (Geserick *et al.*, 2008). Active caspase-8 transmits apoptosis either by direct activation of effector caspase-3 or by triggering the intrinsic apoptotic cell death pathway mediated by cleavage of the BH3-Protein Bid. Bim/tBid heterodimers interact directly with Bax and promote Bax insertion in the mitochondria and pore formation (Kim *et al.*, 2009), which results in apoptosome-dependent capase-9 activation. Active caspase-9 is another promoter of caspase-3 activation and apoptotic cell death, executed by cleavage of hundreds of protease targets. Each step in the cell death signaling cascade is controlled tightly by antiapoptotic proteins. The cellular FLICE-like inhibitory protein (cFLIP) and the X-linked inhibitor of apoptosis proteins (XIAPs) are potent caspase inhibitors, and they are able to suppress either extrinsic or intrinsic cell death. cFLIP inhibits active caspase-8 release from the DISC, thereby suppressing TRAIL- or CD95L-induced apoptosis in melanoma (Geserick *et al.*, 2008). In addition, suppression of the caspase-3/caspase-9 inhibitor XIAP by mitochondrial smac or small-molecule smac mimetics (IAP antagonists) also promotes DR-mediated cell death (Geserick *et al.*, 2009).

Clinical Implications

- Recently, rapid progress in melanoma pathobiology has led to guarded optimism about its ultimate control with pharmacologic agents.
- It has become apparent that long-term remissions from metastatic melanoma will likely require innovative combinations of therapies.
- Combining BRAF inhibitors with other agents that synergistically target additional "hallmarks of skin cancer" appears most promising.
- Combination therapies of BRAF inhibitors with death receptor agonists could be "worth a try."

As reported in this issue of *JID*, Berger *et al.* (2014) investigated the potential of BRAF and MEK inhibitors, not only to suppress cell growth but also the impact of these compounds to modulate DR-mediated cell death. This team from Eberle's group mechanistically investigated the role of a pan-BRAF inhibitor (L-779450) and compared it with the $BRAF^{V600E}$ inhibitor vemurafenib during TRAIL-induced apoptosis in melanoma. Prestimulation with L-779450 resulted in an increased TRAIL cell death response, which correlated tightly with the suppression of extracellular signal–regulated kinase (ERK) phosphorylation. This observed decrease in ERK phosphorylation was accompanied by the upregulation of the proapoptotic "BH3-only" Bcl-2 protein Bim, by increased conformationally active Bax protein, and subsequent release of smac, cytochrome *c*, and apoptosis-inducing factor from mitochondria. This led to the activation of caspase-3, which was mediated either

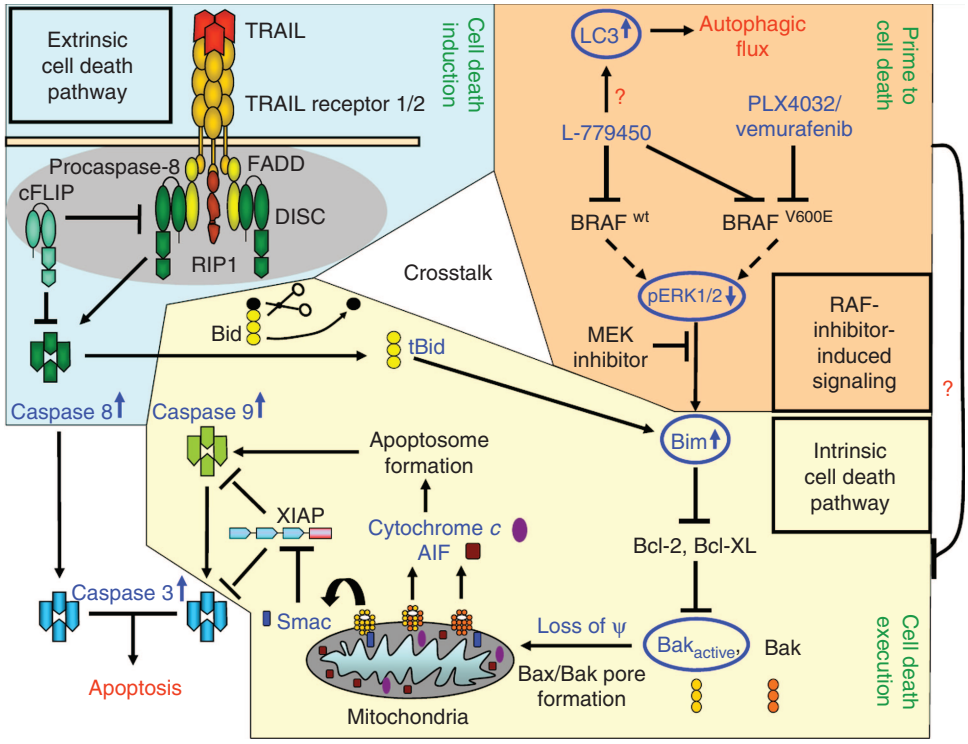

Figure 1. The role of RAF-inhibitor-induced signaling for the crosstalk of extrinsic and intrinsic cell death signaling pathway in melanomas. The influence of the pan-RAF inhibitor (L-779450) and BRAF-specific inhibitor (vemurafenib) for extrinsic and intrinsic cell death pathways is marked in blue color. Consequences of this treatment are marked in red color. Treatment with the pan-RAF-inhibitor L-779450 inhibits both wild-type and mutant BRAF, whereas vemurafenib suppresses only $BRAF^{V600E}$. Treatment with RAF inhibitors results in (**a**) the upregulation of LC3 (autophagy), and (**b**) inhibition of extracellular signal–regulated kinase (ERK) phosphorylation and resulting upregulation of Bim without direct cell death induction ("priming" of melanoma cells to death). Activation of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) death receptors results in procaspase-8 cleavage, subsequent Bid cleavage, and further activation of Bim. This is sufficient to ultimately induce cell death by the inhibition of antiapoptotic Bcl-2 proteins, and subsequent Bax, but not Bak activation. This is sufficient for MOMP, loss of mitochondrial membrane potential (Ψ), and release of cytochrome *c* and apoptosis-inducing factor. In turn, caspase-9 activation in the apoptosome complex as well as release of smac that suppresses X-linked inhibitor of apoptosis protein leads to melanoma cell death. Either TRAIL-mediated activation of caspase-8 or mitochondria-facilitated caspase-9 activation (by Bim-facilitated MOMP) leads to caspase-3 activation and apoptosis execution. The role of the caspase-8 inhibitor cellular FLICE-like inhibitory protein and the impact of BRAF for the regulation of autophagy needs to be substantiated by further experiments but represents an intriguing twist of BRAF-inhibitor resistance in melanoma. However, acute interference with RAF signaling seems to modulate the intrinsic signaling pathway directly, opening a "window of opportunity" to overcome resistance mechanisms by combining BRAF inhibitors with death receptor ligands, autophagy inhibitors, or other compounds that attack the mitochondria and therefore overcome the "primed to death" status of melanoma cells and effectively kill them. MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase. ?, indicates currently unknown interactions suggested by Berger *et al.*, (2014).

by activation of caspase-8 (TRAIL treatment) and/or caspase-9 (after $BRAF^{V600E}$ inhibition). Importantly, functional relevance was demonstrated, because knockdown of either Bim, Bax, or smac rescued the melanoma cells from death, indicating the indispensable role of the intrinsic cell death pathway during sensitization for TRAIL-mediated cell death by BRAF inhibitors (Figure 1, blue-labeled molecules and processes). The investigators then went on and studied an intriguing novel aspect of their results, namely, regulation of the autophagic machinery. Their data give initial hints for increased autophagy, as indicated by the upregulation of LC3 protein and increased autophagic cellular morphology upon BRAF-inhibitor treatment in BRAF-inhibitor-resistant melanoma cells. These data indicate that melanoma cells may signal for increased autophagy and thereby increased cellular recycling mechanisms as an explanation for treatment resistance.

The study by Eberle and colleagues highlights several fascinating insights relevant to treatment. Their first observation is the suppression of ERK phosphorylation and the resulting upregulation of Bim that follows inhibition of RAF signaling. Here, RAF-inhibitor-mediated Bim activation may ''prime the cells to death''. From their study, it becomes clear that Bim activation may be necessary but not sufficient to eliminate RAF-inhibitor-treated melanoma cells. Thus, the mitogen-activated protein kinase (MAPK) pathway activated by RAF proteins is critical for the regulation of intrinsic cell death signaling machinery. The second important finding is that only combined treatment of a RAF inhibitor with TRAIL triggers the activation of Bid and Bim, which then results in the activation of MOMP and subsequent cell death. For clinical investigation, both observations may have relevance for the treatment of melanomas. Altogether the study demonstrates that priming toward cell death, mediated by the suppression of MAPK signaling pathways in combination with activation of TRAIL DRs may be a feasible strategy to avoid RAF-inhibitor resistance. However, this study also raises the critical question: what additional ''hallmarks of skin cancer'' could be targeted to increase the efficacy of melanoma treatment? As mentioned, activation of caspase-8 within the DISC is indispensable for caspase-3 cleavage and activation of the mitochondrial pathway by TRAIL. In this context, the negative regulation of caspase-8 by its direct inhibitor cFLIP with

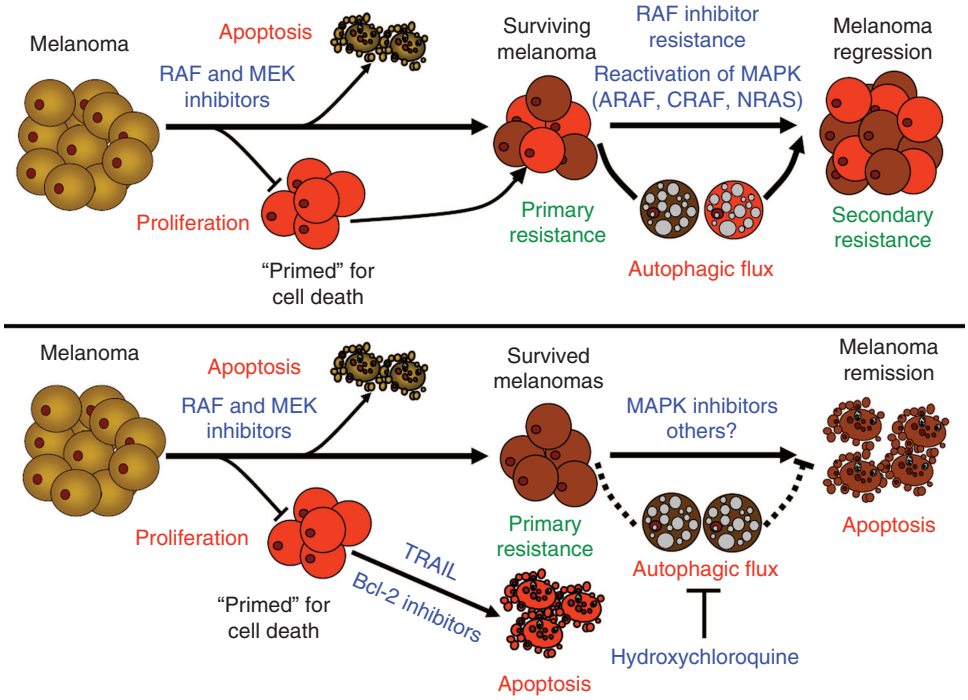

Figure 2. The TRAIL to overcome BRAF-inhibitor resistance: BRAF inhibitor (vemurafenib), pan-RAF inhibitor (L-779450), and MEK inhibitor carry a high clinically measurable potential for melanoma therapy. However, all inhibitors induce only low levels of cell death and rather prime the melanoma cells to cell death. RAF inhibitors suppress cell proliferation substantially but cannot trigger melanoma cell death sufficiently. Without further death stimuli, long-term stimulation with RAF inhibitors therefore promotes development of secondary resistance by alternative activation of other MAPK signaling pathways such as ARAF, NRAS, and CRAF. Furthermore, RAF-resistant melanomas circumvent the cell death priming event by the induction of autophagy. Both reactivation of alternative MAPKs as well as activation of the cellular recycling process of autophagy during RAF-inhibitor treatment can lead to increased melanoma remission. We speculate that RAF and MEK inhibitors as cell death priming compounds that are rapidly followed by direct cell death stimuli such as TRAIL or BH3-mimetics may prove to be highly beneficial for melanomas. Melanoma cells that survive such combination therapies may benefit from combination treatment with the autophagy-inhibitor hydroxychloroquine or with pan-RAF inhibitors that target all MAPK. The overall goal of such therapeutic intervention is to overcome secondary resistance by strong and rapid elimination of all tumor cells without targeting primary cells, facilitated by the use of tumor-specific targeting of mutant BRAF. AIF, apoptosis-inducing factor; cFLIP, cellular FLICE-like inhibitory protein; DISC, death-inducing signaling complex; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; RIP1, receptor-interacting protein 1; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

its different isoforms appears to operate in melanoma (Geserick *et al.*, 2008). Thus, cFLIP can also block tBid–Bim interaction and protect from priming toward cell death, resulting in a lack of BRAF-inhibitor activity. Further studies are required to investigate the impact of cFLIP as a biomarker for sensitivity to activation of the cell death machinery in melanoma whenever RAF/MEK inhibitors are used. In respect to the noted suppression of ERK phosphorylation during BRAF inhibition, novel inhibitors farther downstream, which target MEK or ERK directly, and therefore negatively regulate Bim, may also be a promising strategy to prime melanomas toward cell death. Therefore, compound combinations of BRAF and MAPK inhibitors (for example, trametinib) in combination with DR agonists may represent a useful strategy (Wolchok *et al.*, 2013). An additional possibility that has emerged over the last 5 years is the direct inhibition of antiapoptotic proteins of the Bcl-2 family. So-called BH3-mimetics that activate cell death directly by inhibiting Bcl-2 and Bcl-XL and promoting intrinsic cell death by activation of Bax/Bak were developed and introduced recently (Souers *et al.*, 2013). If Bim is the critical protein that primes melanoma to TRAIL-mediated cell death, a number of treatment strategies become attractive: Compounds that activate the cleavage of Bid (as ''simulated'' by activation of caspase-8 during TRAIL stimulation), MEK or ERK inhibitors (Morris *et al.*, 2013), or, more directly, BH3-mimetics such as ABT199 that activate the Bax/Bak cell death machinery directly (Souers *et al.*, 2013) could prove beneficial as combination treatments.

Finally, the induction of autophagy was hypothesized in the article by Eberle's group as a mechanism of how melanomas bypass the cell death priming by RAF inhibitors and circumvent cell death. This raises the intriguing possibility that BRAF-resistant melanomas may have the ability to activate protective proautophagic signaling pathways. For example, melanoma cells have high requirements for autophagic flux (Xie *et al.*, 2013). Thus, they may undergo apoptosis upon co-treatment using a BRAF inhibitor in combination with lysosomotropic agents, such as the autophagy-inhibitor hydroxychloroquine (Figure 2). Taken together, it appears that the best strategy to target melanoma cells is with a first therapeutic combination regimen and thereby prevent secondary resistance. Given the spectacular progress in the development of RAF inhibitors, initial treatment with more than one small molecule that inhibits several signaling pathways operative in melanoma appears to be the better ''TRAIL to death'' method. As most of the recent data about the plasticity of tumor cells indicate, it appears highly likely that direct elimination of the tumor cells without allowing time for modulation of cell surface or intracellular modulation of key signaling pathways is the key for long-term success. Thus, potentially long-term remissions from metastatic melanoma may require such innovative combination therapies (Holzel *et al.*, 2013). Undoubtedly, further experimental and clinical studies that investigate primary and secondary resistance to BRAF inhibition in tumor cells as such, or in potential treatment-resistant subpopulations (Roesch *et al.*, 2013), will clarify whether and what combinations of cell death agonists, autophagy inhibitors, or ERK or MEK inhibitors will ultimately become useful.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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