Mitogen-Activated Protein Kinase Inhibition Induces Translocation of Bmf to Promote Apoptosis in Melanoma

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Abstract
Constitutive activation of the mitogen-activated protein kinase (MAPK) pathway is implicated in the development and progression of many human cancers, including melanoma. Mutually exclusive activating mutations in NRAS or BRAF have been identified in ∼85% of melanomas, and components of this pathway have been developed as molecular targets for therapeutic intervention. We and others have shown that inhibition of this pathway with specific small molecule MAPK/extracellular signal-regulated kinase (MEK) inhibitors induces a wide range of apoptotic responsiveness in human melanoma cells both in vitro and in vivo. To define the molecular mechanism underlying variable apoptotic sensitivity of melanoma cells to MEK inhibition, we examined the expression and subcellular localization of Bcl-2 family members in a comprehensive set of human melanoma cell lines. Whereas the proapoptotic protein Bim was activated and localized to the mitochondrial membrane in all cell lines regardless of apoptotic sensitivity, Bmf activation and cytosolic translocation was exclusive to sensitive cells. In resistant cells, Bmf remained sequestered to the cytoskeleton through dynenin light chain 2 (DLC2) binding. Overexpression of Bmf in resistant cells did not enhance apoptosis, whereas expression of mutant BmfΔ69P, which has decreased binding to DLC2, promoted cell death. Expression of BmfΔ69P mutants possessing the Bcl-2 homology 3 (BH3) domain L138A, which impairs BH3 interactions, did not enhance apoptosis in resistant cells. RNA interference targeting Bim and Bmf provided protection from apoptosis induced by MEK inhibition. These results show a novel role for Bmf in promoting apoptosis and provide insight into the mechanism of apoptotic resistance to MEK inhibition in melanoma.

Introduction
Skin cancer is the most common malignancy in the United States, representing nearly one third of all newly diagnosed cancers. Melanoma accounts for only 4% of all skin cancers but is responsible for 79% of skin cancer deaths (1). During the last 20 years, the incidence of melanoma has more than tripled in Caucasian Americans with an estimated 62,480 new cases diagnosed in 2008 (1). Melanoma is the most common cancer in men and women of ages 20 to 29 years and the leading cause of cancer death in women between the ages of 25 to 29 years. In 1940, the lifetime risk of developing melanoma was 1 in 1,500, which contrasts sharply with current estimates of 1 in 61 (1). Currently, the alkylating agent dacarbazine (DTIC) is the only Food and Drug Administration–approved chemotherapeutic agent for treating melanoma. Clearly, current therapies used to treat metastatic melanoma are inadequate, as the 5-year survival rate has remained at <15% for decades with over 8,000 deaths annually (1). With mutually exclusive mutations in NRAS and BRAF, this pathway is constitutively activated in most cases of sporadic malignant melanomas (2) and several clinical trials of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)–targeted therapies in melanoma have focused on exploiting the dependence of these tumors on MAPK signaling. Additionally, ∼50% of those with BRAF mutations also have AKT3 amplification or PTEN loss, resulting in deregulation of AKT signaling (3).

Recent in vitro data suggest that MAPK and AKT signaling promotes melanoma cell survival through the regulation of Bcl-2 protein family members (4–6), which are essential regulators of the apoptotic pathway. The antiapoptotic members Bcl-2, Bcl-XL, Mcl-1, Bcl-w, and Bfl-1 each possess four domains termed Bcl-2 homology (BH) domains and are often overexpressed in many cancer types, including melanoma. They regulate the release of cytochrome c from the mitochondria by sequestering proapoptotic Bcl-2 family members. The proapoptotic members fall into two subgroups: those containing BH1–BH3 domains (Bak, Bax, and Bok) and those possessing BH3 domains only (Bad, Bim, Bmf, Bik, Hrk, Bid, Puma, and Noxa). Upon release from antiapoptotic members (e.g., Bcl-2), Bax and/or Bak oligomerizes to induce release of apoptosis-promoting proteins (including cytochrome c) from the mitochondrial intermembrane space. In response to various death-promoting stimuli, BH3-only members are activated by multiple means, including posttranslational modification, transcriptional up-regulation, and subcellular localization (7). BH3-only proteins exhibit selective binding affinities for antiapoptotic Bcl-2 proteins; therefore, activation of two or more can enhance apoptosis depending on the repertoire of antiapoptotic proteins that are present (8).

Increasing evidence suggests that BH3-only members act as sentinels of cellular stress throughout a cell and are regulated by components of the RAS/MAPK and/or phosphatidylinositol 3-kinase (PI3K)/AKT pathways. BAD is a proapoptotic BH3-only member of the Bcl-2 family of proteins that heterodimerizes with and antagonizes prosurvival proteins, such as Bcl-2 and Bcl-xL. This interaction triggers the release of cytochrome c from the mitochondria, which activates a caspase cascade leading to apoptosis (7). Several kinases, including AKT, p70S6K, PKA, c-Jun NH2 kinase (JNK), and RSK (a direct downstream target of ERK), have been

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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shown to phosphorylate and inactivate BAD, thereby promoting survival (4, 9–13). In pancreatic cancer cells, RSK activates the transcription factor cAMP-responsive element binding protein, which promotes survival by increasing the expression of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 (14). The proapoptotic protein Bmf is transcriptionally repressed by both the RAS/MAPK and PI3K/AKT pathways in breast cancer cells (15). In addition, ERK directly phosphorylates the proapoptotic protein Bim, which leads to its rapid degradation via the proteasome pathway (16, 17).

In this study, we classified a comprehensive panel of human melanoma cell lines as either sensitive (>30% cell death) or resistant (<30% cell death) to MEK inhibition to determine the mechanism of resistance to cell death in this context. After MEK inhibition, we compared the expression, activation, and subcellular localization of Bcl-2 family members between resistant and sensitive cell lines to identify potential differences.

Materials and Methods

Cell culture. Human melanoma cell lines C8161 (18) and CHL-1 (wild-type (wt) BRAF and NRAS; ref. 19), A375, C82, CACL, LOX IMVI, M14-Mel, MALME-3M, SK-MEL-5, SK-MEL-28, UACC-62, and UACC-257 (BRAF mutants)3 and SK-MEL-2, SK-MEL-103, and SK-MEL-147 (NRAS mutants) were cultured in RPMI 1640 (Life Technologies) supplemented with 5% fetal bovine serum (HyClone). Human neonatal epidermal melanocytes (Cascade Biologies) were maintained in Medium 254 supplemented with melanocyte growth factors (Cascade Biologies).

Reagents. The MEK inhibitor CI-1040 (PD184352) was obtained from Pfizer Global Research and Development. PD98059, an additional MEK inhibitor, was purchased from Cell Signaling Technology. The BH3 mimetic ABT-737 along with an inactive control molecule was acquired from Abbott. The caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK, and Z-IETD-FMK were purchased from Cell Signaling Technology. The BH3 mimetic ABT-737 along with an inactive control molecule was acquired from Abbott. The caspase inhibitors Z-VAD-FMK, Z-DEVD-FMK, and Z-IETD-FMK were purchased from Becton Dickinson. Drugs were dissolved in DMSO, aliquoted, and stored at –20°C.

Drug treatments and cell viability. All cell viability assays were initiated 24 h after melanocyte or melanoma cell line seeding and were carried out for the times indicated. Single-treatment dosing with CI-1040 (2 μmol/L) was based on titration studies examining phosphorylated ERK levels and minimal melanocyte toxicity. PD98059 required a daily dosing schedule of 20 μmol/L to inhibit ERK phosphorylation. DMSO-treated controls were vehicle control samples that were treated with an equal volume of DMSO. Cell viability and apoptosis were verified by multiple methods, including ViaCount (Guava Technologies), Annexin V binding (Guava Technologies), cell cycle analysis with propidium iodide staining (Roche), and 4,6-diamidino-2-phenylindole staining, as previously described (5). Samples were prepared as per the manufacturers’ specifications at select time points and assayed promptly. Experiments were done in triplicate, and data are presented as mean ± SE.

Subcellular fractionation. Initial mitochondrial and cytosolic fractionation was carried out with M14-MEL and SK-MEL-28 cells over 0, 48, and 72 h after CI-1040 treatment using a commercially available mitochondrial isolation kit (Pierce). Subsequent cytosolic fractional isolations for additional cell lines were carried out in a similar manner using the nuclear and cytosolic fraction kit (BioVision) over multiple time points. Each protocol was followed as per the manufacturers’ instructions, with the exception of transferring the cell pellets to 2-mL microcentrifuge tubes after the first centrifugation followed by washing the cell pellets thrice therein with ice-cold PBS to adequately remove residual trypsin.

Western blotting. For direct immunoblot analysis, adherent and nonadherent cells from each plate or well were lysed in SDS sample buffer and boiled before SDS-PAGE. Protein concentration was determined using the Bio-Rad D6 Protein Assay (Bio-Rad) as per the manufacturer’s specifications. Typically, 30 μg of protein were loaded per well in 4% to 20% gradient polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose (Bio-Rad). Equal volumes of 2× SDS sample buffer was added to native lysates from subcellular fractions, followed by boiling and SDS-PAGE, followed by immunoblotting. For a complete list of all antibodies used, see supplementary data.

Stable Bmf and Bim constructs. V5 NH2 terminal–tagged and wt Bmf and Bim were cloned from M14-Mel cells by reverse transcription–PCR (RT-PCR) into pCB8/GW/TOPO (Invitrogen) and sequence verified with Genbank NM_033503 for Bmf and Genbank NM_138621 for Bim. PCR-based site-directed mutagenesis was used to generate Bmf mutants. Through LR Clonase reactions (Invitrogen), these genes were then recombined into a Gateway-compatible modified lentiviral vector, pDEST-FG12-cmv, for subsequent expression analysis. The pDEST-FG12-cmv vector has been described (20). This vector also harbors an independent GFP reporter gene, allowing easy detection of infection efficiency. Lentiviral production and infections were carried out as previously described (21). Viral gene delivery was confirmed by Western blot analysis. Details regarding primer sequences, cloning strategies, and lentiviral infection are available upon request.

Stable prosurvival Bcl-2 family member constructs. HA NH2 terminal–tagged and wt Bcl-xL and Bcl-2 were cloned from normal human epidermal melanocyte (NHEM) cells by RT-PCR into pCB8/GW/TOPO (Invitrogen) and sequence verified with Genbank NM_009743 for Bcl-xL and Genbank NM_004049 for Bcl-2. Bcl-2 and Mcl-1 cDNAs (Origene) were PCR amplified and cloned into pCB8/GW/TOPO (Invitrogen) and sequence verified with Genbank NM_006653 for Bcl-2 and Genbank NM_021960 for Mcl-1. These genes were then recombined into the pDEST-FG12-cmv lentiviral vector using LR clonase (Invitrogen) for subsequent viral production, infection, and stable expression (21). Infection efficiency was verified by GFP expression, and gene expression was confirmed by Western blot analysis. Primer sequences and cloning strategies are available upon request.

Stable Bmf and Bim short hairpin RNA constructs and Bad short interfering RNA. Short 19-bp to 22-bp Bmf (Genbank NM_033503 nucleotides 326-344, 116-134, 213-235, and 393-414) and Bim (Genbank NM_138621 nucleotides 436-454 and 820-841) specific oligos (IDT) were annealed and cloned into the lentiviral vector KHI-LV (22), which contains an H1 promoter and independent GFP expression driven by a ubiquitin C promoter. An additional scrambled short hairpin RNA (shRNA) was designed and used as a control. Subsequent viral production and infection efficiency was verified by GFP expression, whereas stable shRNA potency was determined by Western blot analysis. Short hairpin oligo sequences and cloning strategies are available upon request. SMARTpools (5 μmol/L; Dharmacon) targeting Bad were electroporated (200 V, 750 μF in Opti-MEM medium) into 5 × 106 melanoma cells using a Gene Pulser II (Bio-Rad), and knockdown was verified by Western blot at the times indicated.

RT-PCR analysis. RNA was isolated (Invitrogen) from CI-1040–treated and untreated melanoma cell lines over times indicated. RT (Invitrogen) of 1 μg of total RNA was followed by 25 cycles of PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Bmf specific primers (IDT). The following primers were used to generate 425-bp (GAPDH) and 254-bp (Bmf) fragments: GAPDH Fwd primer 5’-CATCACCAGCGCAAGTTCAAT-3’, GAPDH Rev primer 5’-TACTCAGCCACGATCACC-3’, and Bmf Fwd primer 5’-GGCTATGGCTTCTCTCC-3’, Bmf Rev primer 5’-TACC- TAGGGCTGCCC-3’.

Results

Melanoma cell lines exhibit variable sensitivity to the MEK inhibitor PD184352 (CI-1040; Fig. 1A). It has been reported that the response of melanoma cells to MEK inhibition is largely due to BRAF (sensitive) and NRAS (resistant) mutation status (23, 24). To define the mechanism underlying the variable apoptotic response of melanoma cells to MEK inhibition, 15 human melanoma cell lines, along with NHEM cells, were treated with the highly specific small molecule MEK inhibitor CI-1040 (25, 26). NHEM and melanoma cells that possess wt NRAS and BRAF are resistant

http://www.sanger.ac.uk/genetics/CGP/Studies/
to the apoptotic effects of MAPK inhibition, whereas variable apoptotic sensitivity was not significantly different between BRAF, BRAF/PTEN, and NRAS mutant cell lines (Fig. 1A). CI-1040 potently inhibited ERK phosphorylation for the duration of the study (Fig. 1B). Although baseline phosphorylated ERK levels were variable, there was no correlation between cell death and ERK activation. CI-1040 promotes G1 cell cycle arrest in NHEM and melanoma cells, followed by significant apoptosis in sensitive melanoma cells by 72 hours (Fig. 1C).

Melanoma cells exhibit variable sensitivity to the BH3 mimetic ABT-737. It was previously reported that inactivation of the BH3-only protein Bad by MAPK signaling promotes survival in melanoma cells (4). The small molecule BH3-mimetic ABT-737 mimics Bad by binding to the same subset of Bcl-2 prosurvival proteins Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or Bfl-1 (27). Unlike Bad, however, ABT-737 cannot be inactivated by MAPK signaling. Therefore, ABT-737 functions in a similar manner as constitutively active Bad and was used to further evaluate the role of Bad in promoting apoptosis in melanoma cells. ABT-737 dosing was determined by single-agent titration ranging from 100 nmol/L to 5 μmol/L on 10 melanoma cell lines for 72 hours. The enantiomer (the stereoisomer having the mirror image of the
dimethylaminoethyl group) was used as a loss-of-function control (27). The range wherein the enantiomer had negligible cytotoxic effects and ABT-737 induced varying degrees of cytotoxicity was between 1 and 2.5 μmol/L for most of the cell lines (Fig. 2A and data not shown). Single and combination treatments with ABT-737 and the MEK inhibitor PD98059 were carried out with a final concentration of 2.5 and 20 μmol/L, respectively. As a control, 2.5 μmol/L enantiomer was also tested, and the resulting cytotoxicity was subtracted from the corresponding ABT-737–induced cytotoxicity. DMSO was used as a control for PD98059. ABT-737 exhibited significant single-agent activity in many of the cell lines with >50% cell death in CHL-1 and SK-MEL-103 (Fig. 2A). However, the sensitivity profile for ABT-737 did not mirror that of the MEK inhibitors. The CHL-1 cell line was highly resistant to MEK inhibition but was sensitive to ABT-737, and the M14-MEL cell line was highly sensitive to MEK inhibition but relatively resistant to ABT-737. Whereas this shows that the apoptotic machinery is intact and resistance to MEK inhibition is not due to defects in the apoptotic pathway (e.g., loss of bak or bax functionality), it suggests that cell death induced by MEK inhibition is not mediated by the BH3-only protein Bad.

Apoptosis of melanoma cells induced by MEK inhibition does not require the BH3-only protein Bad. To further examine the requirement of Bad in apoptosis induced by MEK inhibition, M14-MEL and MALME-3M cells were transfected with synthetic short interfering RNA (siRNA) SMARTpools to specifically reduce Bad expression by RNA interference (RNAi). Expression of Bad was assessed by immunoblot analysis over time after transfection. Levels of Bad protein were effectively reduced at >90% in both cell lines by 120 hours posttransfection compared with the levels expressed in cells transfected with the control siRNAs (Fig. 2B). Cells were treated with CI-1040 48 hours after transfection and assessed for cell death after 72 hours of treatment (120 hours after transfection). Loss of Bad expression reduced apoptosis by only ~1.2-fold in both cell lines, suggesting that Bad is not required for apoptosis induced by MEK inhibition in melanoma cells (Fig. 2B).

**Figure 2.** Biological effects of the Bad-like BH3 mimetic ABT-737 and Bad RNAi in melanoma cells. A, apoptotic sensitivity of melanoma cell lines to ABT-737 alone and in combination with the MEK inhibitor PD98059. Cells were treated in triplicate with either the inactive enantiomer, 2.5 μmol/L ABT-737, DMSO, or 20 μmol/L of the MEK inhibitor PD98059 (added every 24 h) for 72 h. Apoptosis (% sub-G1 fraction) was determined by cell cycle analysis using flow cytometry. Data were normalized to the control and expressed as the mean ± SE. B, analysis of the requirement of the BH3-only protein Bad in CI-1040–induced apoptosis in M14-MEL and MALME-3M cell lines. Cells were treated with DMSO or 2 μmol/L CI-1040 at 48 h after transfection for a duration of 72 h (120 h posttransfection). The arrow indicates the start of CI-1040 treatment. Apoptosis was determined as described in A. Data were normalized to the control and expressed as the mean ± SE. C, untransfected; O, siCtrl; Δ, siBad. Immunoblots show reduced expression of Bad after transfection of siRNA SMARTpool oligonucleotides targeting Bad (Dharmacon).
explore the mechanism of resistance to MEK inhibition, comparative expression analysis of Bcl-2 and IAP family members, which tightly regulate cell survival, in a subset of both sensitive and resistant melanoma cell lines was performed. Immunoblot analysis of apoptotic regulatory factors in eight melanoma cell lines treated with CI-1040 (+) or DMSO (-) for 72 h, and whole-cell lysates were prepared. Western blotting was performed using the antibodies indicated to the left of the blots. Puma shows two isoforms, α and β, as does Bim, EL and L, which are indicated to the right of their corresponding blots. A representative α-tubulin reprobed blot is the loading control (bottom).

Figure 3. Effect of CI-1040 on Bcl-2 and IAP family member proteins. The cell lines indicated above the blots were treated with either DMSO (-) or 2 μmol/L CI-1040 (+) for 72 h, and whole-cell lysates were prepared. Western blotting was performed using the antibodies indicated to the left of the blots. Puma shows two isoforms, α and β, as does Bim, EL and L, which are indicated to the right of their corresponding blots. A representative α-tubulin reprobed blot is the loading control (bottom).

Table: Table 1

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MEK inhibition in several cell lines, but this expression did not correlate with resistance. Additionally, IAP expression did not influence apoptotic sensitivity (Fig. 3). High expression of the proapoptotic multidomain proteins Bax and Bak was observed, suggesting that there is no disconnection with proximal death effectors in these melanoma cell lines. Upon MEK inhibition, p53 responsive proapoptotic proteins Noxa and Puma β expression is reduced in several cell lines (Fig. 3). ERK directly phosphorylates and targets Bim for proteolytic degradation (17), and as expected, inhibition of MEK and subsequent inhibition of ERK leads to a dramatic increase in Bim levels (Fig. 3). Interestingly, active Bim is present in all cell lines in
response to MEK inhibition regardless of the amount of cell death induced.

Comparative compartmental analysis between a sensitive and resistant cell line reveals differences in Bmf localization. Cellular localization of Bcl-2 family members may be more relevant to cell survival than overall expression, as many of these proteins are inactivated through sequestration. To interrogate possible differences between a CI-1040–sensitive (M14-MEL) and CI-1040–resistant (SK-MEL-28) cell line, subcellular fractions were collected from untreated and CI-1040–treated cells over time and compared by immunoblot (Fig. 4A). Bim rapidly accumulates to the outer mitochondrial membrane in both cell lines, but cytosolic accrual of Bmf was exclusive to the sensitive M14-MEL cell line (Fig. 4A). Analysis of whole-cell lysates revealed that expression of Bmf remains relatively constant over time in each cell line (Fig. 3). Whereas nearly all Bcl-2 family members were analyzed in this context, only Bmf localization was dramatically divergent. Further compartmental analysis of Bmf localization in additional cell lines with variable apoptotic sensitivities revealed that Bmf cytosolic localization correlated exquisitely with cell death (Fig. 4B). Resistant cell lines, such as C8161 and SK-MEL-28, retain Bmf in the cytoskeletal compartment. Conversely, Bmf is released into the cytosolic fraction, which is proportional to levels of apoptosis, in CI-1040–sensitive cell lines (Fig. 4B). Interestingly, MEK inhibition results in enhanced Bmf transcription in melanoma cells, regardless of mutation status or apoptotic sensitivity (Fig. 4C).

Apoptosis of melanoma cells induced by MEK inhibition requires the BH3-only proteins Bim and Bmf. To further define the roles of Bim and Bmf in promoting apoptosis, shRNAs were stably expressed in melanoma cells using a lentiviral-mediated approach. The KH1-LV lentivirus (22), which coexpresses GFP driven by the UbC promoter, was VSV-G pseudotyped and used to infect the sensitive cell line M14-MEL. Because Bim is not detectable when MEK is active, levels of Bim were assessed in the presence of MEK inhibition. Infected M14-MEL cells were

Figure 4. Localization of Bmf correlates with resistance to MEK inhibition in a panel of melanoma cell lines. A, a comparison of the cytosolic (Cyto) and mitochondrial (MT) fractions between a CI-1040–sensitive (M14-MEL) and CI-1040–resistant (SK-MEL-28) cell line reveals differences in Bmf localization. Cells were treated with DMSO (0 h) or 2 μmol/L CI-1040 for the times indicated. Representative Western blots of fractionated lysates probed with antibodies directed against the proteins indicated. The blots were reprobed with VDAC as a loading control and confirmation of purity of the mitochondrial fraction. B, correlation between apoptotic sensitivity of NHEMs and melanoma cell lines to CI-1040 and cytosolic Bmf protein levels. Cells were treated in triplicate with either DMSO or 2 μmol/L CI-1040 for 60 h and apoptosis (% sub-G1 fraction) was determined by cell cycle analysis using flow cytometry. Columns, mean; bars, SE. Duplicate samples were treated with 2 μmol/L CI-1040 for the times indicated, and the cytosolic fraction was isolated. Western blots of fractionated lysates probed with an antibody directed against Bmf (top blot). The blots were reprobed with α-tubulin as both a loading control and confirmation of purity of the cytosolic fraction (bottom blot). C, up-regulation of Bmf RNA levels after treatment with CI-1040. RT-PCR products for Bmf and GAPDH were derived from DMSO treated cells (0 h) or cells treated with 2 μmol/L CI-1040 for the times indicated. The cell lines analyzed are indicated above each corresponding gel.
treated with DMSO or CI-1040 for 72 hours, and expression of Bim and Bmf was assessed by immunoblot analysis. Protein levels of Bim in the CI-1040–treated samples were reduced by 20% and 80% using shRNA1 and shRNA2, respectively, compared with the levels expressed in cells infected with the scrambled control shRNA lentivirus (Fig. 5A). Bmf protein levels were unchanged when only one shRNA target was used, but a pool of three shRNAs reduced Bmf protein levels by 64% compared with the levels expressed in cells infected with the control shRNA lentivirus (Fig. 5A). Reduced expression of either Bim or Bmf dramatically reduced CI-1040–directed apoptosis in these cells (Fig. 5A), whereas reduction of other BH3-only proteins, such as Bad or Bid, did not (Fig. 2B and data not shown). These data provide compelling evidence that both Bim and Bmf are required for promoting apoptosis in response to MEK inhibition in melanoma cells.

**Cl-1040–directed apoptosis shows partial caspase dependence.** Truncated Bid, an effector of the extrinsic apoptotic pathway, was present in M14-MEL (Fig. 4A) and other sensitive cell lines (data not shown) during active apoptosis. To determine the relative contribution of the extrinsic pathway in promoting apoptosis induced by MEK inhibition, caspase inhibitors with differing specificities were used. The pan caspase inhibitor Z-VAD-FMK and the caspase-3–directed Z-DEVD-FMK provided dose-differing specificities were used. The pan caspase inhibitor Z-VAD-FMK (28, 29) as dynein light chain 2 (DLC2), is crucial for Bmf to promote apoptosis (28, 29). To promote Bmf disassociation from DLC2, we generated BmfA69P (28) and BmfA69P/L138A mutants and evaluated their effect on cell death in response to MEK inhibition. Overexpression of either Bmf mutant in the absence of CI-1040 had no effect on cell death (Fig. 6), but upon MEK inhibition, only BmfA69P dramatically enhanced apoptosis in resistant SK-MEL-28 cells to a level comparable with the sensitive cell line M14-MEL (Fig. 6A). Increased amounts of cleaved poly(ADP-ribose) polymerase (PARP), an indicator of caspase-3 activation and apoptosis, was observed in CI-1040–treated SK-MEL-28 cells expressing BmfA69P compared with the same cells expressing wt Bmf and was comparable with CI-1040–treated M14-MEL cells (Fig. 6A). BmfA69P and BmfA69P/L138A mutants each localize to the cytosolic fraction in SK-MEL-28 cells (data not shown), but the BH3 domain impaired BmfA69P/L138A mutant does not promote apoptosis. Therefore, the apoptotic effects of Bmf require cytoskeletal disassociation and an intact BH3 domain.

Figure 5. Bmf or Bim RNAi protects melanoma cells from CI-1040–induced apoptosis. A, after stable lentiviral shRNA expression targeting Bim, Bmf, or scrambled control (Ctrl) in the sensitive M14-MEL cell line, cells were treated with 2 μmol/L CI-1040 or equal volume DMSO for 72 h, and apoptosis (% sub-G1 fraction) was determined by flow cytometry. Columns, mean; bars, SE. Two different shRNAs with different efficiencies were used. Reduced expression of Bim and Bmf was confirmed by Western blot analysis with the antibodies indicated to the left of each panel. Blots were stripped and reprobed with α-tubulin as a loading control. B, apoptotic sensitivity of M14-MEL cells treated with 2 μmol/L CI-1040 and increasing levels of the pan caspase inhibitor ZVAD-FMK (○), the caspase-3 inhibitor Z-DEVD-FMK (●), or the caspase-8 inhibitor Z-IETD-FMK (▲). Apoptosis was determined as described in A. Points, mean; bars, SE.

**Prosurvival Bcl-2 family member overexpression and antagonism affect melanoma survival.** To evaluate the contribution of Bcl-2 prosurvival members in resistance to MEK inhibition, lentiviral vectors (21, 22) were used to deliver and express Bcl-2, Bcl-xL, Bfl-1, and Mcl-1. Overexpression of any of these proteins in the sensitive cell line M14-MEL resulted in significant protection from apoptosis induced by CI-1040 at 72 hours (Fig. 6 and data not shown). PARP cleavage was not observed in Mcl-1 or Bcl-xL overexpressing cells (Fig. 6). Expression of Bcl-xL in additional sensitive cell lines A375 and MALME-3M efficiently abrogated apoptosis (data not shown). Apoptosis induced by MEK inhibition is significantly enhanced by RNAi-mediated reduction in Bcl-2, Bcl-xL, or Mcl-1 levels in SK-MEL-103 and SK-MEL-147 cells (21). This shows that these cell lines can effectively disseminate intrinsic apoptotic signals when the protective effects of Bcl-2 prosurvival members are removed. However, because no correlation between endogenous expression (Fig. 3) and apoptotic
sensitivity was observed, it is unlikely that these proteins are responsible for the variable sensitivity to MEK inhibition observed in melanoma cells (Fig. 1A).

Discussion

By analyzing a large panel of melanoma cells with varying sensitivity to MEK inhibition, we have identified a role for the proapoptotic BH3-only protein Bmf in promoting cell death in response to MEK inhibition. Striking differences in Bmf activation and localization distinguished sensitive and resistant cell lines exquisitely. Unlike Bmf, Bim translocation to the outer mitochondrial membrane occurred in all melanoma cell lines regardless of sensitivity or resistance to MEK inhibition. Interestingly, both Bmf and Bim are necessary but not sufficient to promote significant apoptosis. These data suggest that strategies that induce the activation of additional proapoptotic proteins or those designed to abrogate the protective effects of most of the prosurvival proteins are more likely to achieve success in combination with MEK inhibition in melanoma.

There has been considerable debate regarding the mechanism underlying variable apoptotic sensitivity of melanoma cells to MEK inhibition, most notably NRAS or BRAF mutation status. Recently, it was reported that the IC50 values of current generation MEK inhibitors were substantially lower in BRAF mutant melanoma cell lines compared with those harboring activating NRAS mutations, suggesting that mutation status is predictive of response (24). We analyzed the apoptotic response of an inclusive melanoma cell line panel in the presence of complete MEK inhibition for the duration of our studies (Fig. 4B). The results show that the apoptotic sensitivity of melanoma cells correlates with Bmf subcellular translocation in response to MEK inhibition regardless of NRAS or BRAF mutation status. Interestingly, treating this cell line panel with doses of CI-1040 that resulted in only modest reductions in ERK phosphorylation yielded no cell death even in the most sensitive cell lines (data not shown). Additionally, removal of CI-1040 treatment after 24 hours allows all cell lines to completely recover and avert cell death. Recent evidence indicates that Bim is quickly phosphorylated and degraded in response to MAPK reactivation (16). Collectively, it is essential to completely block MEK/ERK signaling for a significant amount of time (e.g., 48–96 hours) to elicit efficient cell death. However, the most resistant cell lines (e.g., C8161 and SK-MEL-28) remain viable even after 144 hours of continuous MEK inhibition (data not shown).

A recent study using the MEK inhibitors U0126 and CI-1040 found that, whereas phosphorylated ERK may be a valid marker for assessing MEK inhibition, it did not correlate with inhibition of melanoma cell growth or BRAF mutational status (18). A separate study using anthrax lethal toxin–induced MEK inhibition reported that melanoma cells harboring mutant BRAF are more sensitive...
compared with those containing mutant NRAS. However, there were exceptions to this finding, as two mutant BRAF cell lines, SK-MEL-5 and A2058, had similar levels of sensitivity as the NRAS mutant cells and a cell line for wt BRAF, SK-MEL-31, was highly sensitive to MEK inhibition (23). In addition, SK-MEL-5 and SK-MEL-28 are highly resistant to apoptosis induced by anthracyl lethal toxin (5), which is similar to what we observed with CI-1040.

Previous studies have suggested that inactivation of the BH3-only protein Bad by MAPK signaling promotes survival in melanoma cells and that interfering with this signaling may represent a tumor-specific target (4). Our findings indicate that Bim and Bmf, but not Bad or Bid, are functionally required to induce apoptosis after MEK inhibition. It has previously been reported that Bim, Bid, and Puma bind all of the prosurvival proteins, whereas the remaining BH3-only proteins are more restricted. Whereas Bad and Bmf were reported to only interact with Bcl-2, Bcl-xl, and Bcl-w (30), recent data suggests that Bmf can also interact with Mcl-1 and Bfl-1, albeit with different affinities (8, 31). Furthermore, Bmf was first identified in a screen for binding partners to Mcl-1, and the BH3 domain in Bmf is most similar to that in Bim (28).

Several studies have described a role for Bim and Bmf in cell death mediated by specific processes. For example, Bmf has been reported to play a role in apoptosis mediated by histone deacetylase inhibitors (32) and both Bim and Bmf are implicated in the cell death response to transforming growth factor-β (33) and arsenic treatment (34). In addition, Bim and Bmf play key roles in mammary epithelial anoikis and morphogenesis (15). Here, we show that Bim and Bmf mediate cell death in response to MEK inhibition in melanoma. Interestingly, RNAi targeting either Bim or Bmf is sufficient to rescue cells from cell death in some models, whereas reduction in both proteins is required to significantly suppress apoptosis in other systems. These discrepancies are likely due to differences in cell types and/or expression of prosurvival Bcl-2 family members.

Expression of the p53-responsive BH3-only protein Puma has been reported to be reduced during melanoma progression and low Puma expression correlates with poor prognosis (35). Whereas no significant difference in Puma expression was observed between cell lines, loss or reduced expression occurred over the CI-1040 treatment time course (Figs. 2 and 3). Additionally, expression of another p53-responsive BH3-only protein Noxa is dramatically reduced or lost upon CI-1040 treatment. Taken together, these data suggest that these proteins play little or no role in sensitivity or resistance to MEK inhibition, as their expression is reduced upon MEK inhibition. However, it is probable that these active BH3-only proteins are responsible for apoptosis induced by ABT-737.

Two distinct models of how BH3-only proteins induce apoptosis have been proposed (36, 37). Whereas it is accepted that Bax or Bak are required for cell death, it is unclear whether BH3-only proteins activate Bax or Bak directly or indirectly. Our results are consistent with the direct activation model that requires a functional activator (Bim) and sensitizer (Bmf) BH3-only pair to induce apoptosis. Bim activation occurs rapidly and robustly upon MEK inhibition in all cell lines examined regardless of the level of apoptosis induced (Figs. 3 and 4), but cell death occurs only when an additional BH3-only member (Bmf) is concurrently active. Bim and Bmf RNAi data are supportive of this model, because reducing the expression of either one dramatically blocks cell death (Fig. 5A). Bim binds to and antagonizes all Bcl-2 prosurvival members, whereas Bmf binds to a smaller subset with different affinity (8). Further investigation may distinguish which model, if either, is correct.

Our results suggest that a MEK-independent mechanism of retaining Bmf in the cytoskeletal compartment (e.g., DLC2) is lacking in the sensitive melanoma cell lines and is a major contributing factor to apoptosis that is directed by MEK inhibition. Several kinases, including JNK1 (MAPK8), have been implicated in the regulation and sequestration of Bmf to DLC2 (38). Combined targeting of these kinases or upstream components with MEK inhibition may expand the utility of MAPK as a therapeutic target. Single-agent MEK inhibition must be nearly complete to initiate apoptosis in even the most sensitive cell lines. Results from early-phase clinical trials have shown that tolerable doses of current generation MEK inhibitors PD 0325901 and AZD6244 reduce phosphorylated ERK levels at ~84% and ~79%, respectively (39, 40). Additionally, the greatest tumor reduction (70%) in a melanoma patient, whose tumor contained an NRAS mutation, had undetectable phosphorylated ERK levels in the tumor after sustained daily AZD6244 treatment (39). Complete and sustained MEK inhibition is unlikely to be achieved unilaterally in vivo due to an increase in frequency and severity of side effects upon dose escalation and bioavailability of these inhibitors in organs in which melanoma commonly metastasizes (e.g., brain and liver). Therefore, by understanding the mechanism driving cell death in response to MEK inhibition in melanoma, logical combinatorial therapies can be developed. Consistent with this idea, a novel BH3 mimetic, TW-37, which binds Mcl-1, Bcl-xl, and Bcl-2, in combination with CI-1040, showed synergy in a MEK inhibitor–resistant melanoma cell line in vivo (21). Despite its inability to antagonize Mcl-1, which all of the melanoma cells examined express (Fig. 3), ABT-737 had a relatively high single-agent activity in this panel of melanoma cell lines, and when combined with a less potent MEK inhibitor (PD98059), cell death was enhanced (Fig. 2). Our results provide new insights into how MEK inhibition induces apoptosis in melanoma cells. Future studies will determine the mechanism by which Bmf remains sequestered in melanoma cells resistant to MEK inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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