A Novel BH3 Mimetic Reveals a Mitogen-Activated Protein Kinase–Dependent Mechanism of Melanoma Cell Death Controlled by p53 and Reactive Oxygen Species

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Abstract

The RAS/BRAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway is emerging as a key modulator of melanoma initiation and progression. However, a variety of clinical studies indicate that inhibiting the MAPK pathway is insufficient per se to effectively kill melanoma cells. Here, we report on a genetic and pharmacologic approach to identify survival factors responsible for the resistance of melanoma cells to MEK/ERK antagonists. In addition, we describe a new tumor cell–selective means to bypass this resistance in vitro and in vivo. By generating a panel of isogenic cell lines with specific defects in the apoptotic machinery, we found that the ability of melanoma cells to survive in the absence of functional MEK relies on an ERK-independent expression of the antiapoptotic factor Mcl-1 (and to a lesser extent, Bel-xL and Bel-2). Using computer-based modeling, we developed a novel Bcl-2 homology domain 3 (BH3) mimetic. This compound, named TW-37, is the first rationally designed small molecule with high affinity for Mcl-1, Bel-xL, and Bel-2. Mechanistic analyses of the mode of action of TW-37 showed a synergistic tumor cell killing in the presence of MEK inhibitors. Importantly, TW-37 unveiled an unexpected role of the MAPK pathway in the control of reactive oxygen species (ROS). This function was critical to prevent the activation of proapoptotic functions of p53 in melanoma cells, but surprisingly, it was dispensable for normal melanocytes. Our results support that this MAPK-dependent ROS/p53 feedback loop is a point of vulnerability of melanoma cells that can be exploited for rational drug design. (Cancer Res 2006; 66(23): 11348-59)

Introduction

The identification of tumor-associated genetic and epigenetic hallmarks is providing a rational platform for molecularly targeted cancer therapies (1, 2). In particular, the concept that tumor cells may remain dependent on the oncogenes that promote cell transformation is being exploited for the design of more selective anticancer agents (3). However, the identification of targets for drug development is frequently challenged by the complex and heterogeneous background of neoplastic cells. Malignant melanoma is a prime example of an aggressive tumor type containing aneuploid cells, which undergo a plethora of changes in gene expression during malignant transformation (4, 5). The extreme resistance of melanoma cells to standard chemotherapeutic agents, either as single agents or in combination, has hampered the identification of prognostic factors or predictors of treatment response (6, 7).

Further complicating drug design, the apoptotic machinery, particularly the intrinsic or mitochondrial pathway, is defective in aggressive melanoma cells (6, 8). For example, the activation of p53, a main modulator of this pathway, can be compromised by up-regulation of negative regulators (e.g., HDM-2; ref. 9) or by defective positive effectors (e.g., 14-3-3 or p14ARF; refs. 10, 11). Moreover, multiple antiapoptotic members of the Bcl-2 family (primarily Bcl-xL and Mcl-1) can act downstream of p53 to prevent the release from the mitochondria of cytochrome c, Smac, ALF, and other death inducers (6, 8). Additionally, inhibition of caspases can result from the increased expression of several members of the inhibitors of apoptosis proteins (IAP) family (12) and/or by down-regulation of APAF-1, a cofactor of caspase-9 (13, 14). Overexpression of proteins such as SURVIVIN, which act at the interface between cell cycle progression and death, can also contribute to the aggressive phenotype of melanoma cells (8).

It is conceivable that key determinants of melanoma cell survival are acquired in a progressive and independent manner at different stages of tumor development. However, multiple alterations affecting the core of the apoptotic machinery rely on simultaneous transcriptional or posttranslational events (affecting, for example, members of the Bcl-2 and IAP families; refs. 6, 8). Therefore, it is possible that at least some antiapoptotic events are collectively regulated. The identification of such master regulator(s) would provide an ideal target for therapeutic intervention. In this context, the RAS/BRAF/MEK/ERK mitogen-activated protein kinase (MAPK) pathway is raising high expectations for the rational design of more effective anti-melanoma therapies (15–17). This pathway is invariably activated in early-, intermediate-, and late-stage melanomas (see ref. 5 for review), and dysregulated MAPK signaling contributes to the resistance of melanoma cells to a variety of chemotherapeutic agents (16, 18, 19). However, the precise contribution of downstream targets of ERK to melanoma cell survival is not well understood.
In a variety of tumor cell types, ERK can block apoptosis by favoring the transcription and activation of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-xL, and Mcl-1), or by inhibiting proapoptotic factors, such as BimEL or Bad (20–22). BRAF and ERK have also been reported to interfere with events acting downstream of the mitochondria, ultimately preventing the activation of caspase-9 and the execution of cell death (19, 23). However, whereas BRAF, MEK, or ERK inhibitors can efficiently block melanoma cell proliferation (15, 16, 24), the killing activity of these compounds seems limited to selective groups of melanoma cells (25–27). Thus, the inhibition of MEK may be ineffective as a death inducer in melanoma cells lacking BRAF mutations (which constitute the majority of acral or mucosal melanomas and >30% of cutaneous melanomas; ref. 4). Moreover, melanoma clinical trials with farnesyltransferase inhibitors (to block RAS signaling), sorafenib (which inhibits CRAF and BRAF), or the MEK inhibitor PD-0325901 have shown only modest clinical effect as single agents (see ref. 28 for review). Therefore, identifying new compounds that can bypass the resistance to MAPK inhibition could have a major effect in melanoma therapy.

To investigate the interplay between the MAPK pathway and the apoptotic machinery of melanoma cells, here we used lentiviral-driven short hairpin RNAs (shRNA) to generate isogenic lines with specific defects in the apoptotic machinery. This strategy identified Mcl-1, Bcl-xL, and Bcl-2 as critical mediators of the resistance to MEK inhibition. Because no effective synthetic inhibitor of Mcl-1 has been described, we used a computational approach to generate TW-37, the first rationally designed BH3 mimetic able to block Mcl-1, Bcl-xL, and Bcl-2. TW-37 and a MEK inhibitor synergistically killed aggressive melanoma cell lines, with minimal secondary toxicity for normal skin cells (melanocytes, keratinocytes, and fibroblasts). We present a comprehensive characterization of the molecular basis underlying the synergistic interaction between TW-37 and inactive MEK/ERK. Our studies revealed an unexpected tumor cell–selective role of the MAPK pathway upstream of the mitochondria, controlling reactive oxygen species (ROS) production and the activation of proapoptotic functions of p53. Our findings emphasize the power of RNA interference to generate a rational pharmacologic approach to overcome melanoma chemo-resistance.

Materials and Methods

**Cell culture.** The nonmetastatic WM-1366 cell line and the metastatic melanoma lines Malme-3M, UACC-62, G361, UACC-257, and the SK-Mel series (SK-Mel-19, SK-Mel-28, SK-Mel-29, SK-Mel-94, SK-Mel-103, SK-Mel-147, and SK-Mel-173) were cultured in DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Nova-Tech, Inc., Grand Island, NY). Human melanocytes were isolated from human neonatal foreskins as described (29) and maintained in Medium 254 supplemented with 10% fetal bovine serum (Nova-Tech, Inc., Grand Island, NY). Alternatively, mitochondrial and cytosolic cell fractions were obtained from melanoma cells by either scraping or trypsinization and pelleted by centrifugation at 800 g. Protein samples were separated on 12% or 4-20% gradient SDS-PAGE gels and protein bands were visualized by Coomassie blue or silver stain.

**Protein immunoblots.** Protein samples were separated on 12% or 4-20% gradient SDS-PAGE gels and protein bands were visualized by Coomassie blue or silver stain. Protein samples were separated on 12% or 4-20% gradient SDS-PAGE gels and protein bands were visualized by Coomassie blue or silver stain.

**Cell cycle analyses.** Cells were harvested following appropriate drug treatments and fixed with 1 mL of ice-cold 70% ethanol. Cells were washed twice with PBS followed by incubation in a labeling solution of 50 μg/mL propidium iodide (PI; Sigma Chemical, St. Louis, MO). Alternatively, mitochondrial and cytosolic cell fractions were prepared by digitonin extraction as previously described (33). Sixty micrograms of the cytosolic fractions were used for immunoblot analysis of the release of proapoptotic proteins from the mitochondria. α-Tubulin or β-actin were used as loading controls. See Supplementary Information for a list of the antibodies used in this study.

**Stable short hairpin interfering RNA constructs.** Published references were used as a guide to generate short 19-29 hairpins for RNA interference: Bcl-2, nucleotides 500-518, GenBank M13995 (34); Bcl-xL, nucleotides 714-732, GenBank NM000546 (36); p53, nucleotides 611-629, GenBank NM000546 (36); BAK, nucleotides 535-553, GenBank NM001188 (37). Oligos against McI-1 (nucleotides 2345-2361, GenBank NM021960) and BAX

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8 http://sw16.im.med.umnch.edu/software/calc_ki/
(nucleotides 239-257, Genbank NM138761) were generated using the OligoRetriever database.\(^9\) BLAST search was done to ensure at least 4-nucleotide differences with annotated human genes. The corresponding oligonucleotides were annealed and cloned under the control of the H1 promoter into a self-inactivating lentiviral vector (38). The vector was also designed to carry the green fluorescent protein (GFP) reporter gene under control of the human ubiquitin-C promoter to monitor infection efficiency. A scrambled shRNA construct was also designed to be used as a control. Lentiviral infections were done essentially as described elsewhere (38), and the potency and specificity of each construct were determined by protein immunoblotting (see text). Primer sequences, cloning strategies, and transfection and infection strategies are available from the authors upon request.

**Immunofluorescent visualization of activation-dependent conformational changes of BAX.** Cells of interest were seeded onto glass culture slides and treated with TW-37 (5 μM/L) in the presence or absence of U0126 (10 μM/L). Antioxidants were added as indicated. Cells were fixed with 4% formaldehyde at different time points after treatment, permeabilized with 0.2% Triton-X in PBS for 5 minutes, and washed thrice with PBS. Following a 30-minute blocking step in 1% bovine serum albumin, the rabbit polyclonal Bax-NT antibody from Upstate Biotechnology (Lake Placid, NY) was used to visualize conformational changes in BAX following previously described protocols (39, 40). Melanoma cells rounded up and floated immediately after cytochrome c release (data not shown). To avoid for indirect effects on BAX conformation as byproducts of cell death, only adherent cells (corresponding to early apoptotic cells) were analyzed. The percentage of cells with positive staining was estimated using vehicle-treated cells as a reference.

**Detection of ROS production by fluorescent microscopy.** Melanoma cell lines and melanocytes were seeded onto glass culture slides (BD Biosciences, Bedford, MA) and treated with the indicated drugs. At various time points, medium was aspirated, and cells were loaded with 1 μM/L CM-H2DCFDA in PBS for 10 minutes at 37°C in the dark followed by a PBS wash step essentially as previously reported (32); 0.03% H2O2 was used as a reference control for an active ROS inducer. DCF-dependent fluorescence was scored with ImageJ software and expressed with respect to untreated control cells.

**Indirect measurement of oxidized proteins.** Following drug treatments, both adherent and nonadherent cells were collected and subjected to Laemmli extraction. Oxidized proteins were visualized by derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) using the OxyBlot Oxidized Protein Detection kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Following SDS-PAGE separation and standard Western blotting, the resulting DNPH-hydrazone side chains were detected with a specific antibody to the DNP moiety of the protein.

**Melanoma growth in vivo (mouse xenografts).** Athymic Ncr-nu/nu mice (National Cancer Institute) were kept in pathogen-free conditions and used at 8 to 12 weeks of age. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals of the University of Michigan. To analyze localized melanoma growth in vivo, 0.5 × 10\(^6\) GFP-expressing SK-Mel-147 melanoma cells were injected s.c. in both rear flanks (n = 12 tumors per experimental condition). Treatment was initiated on the third day following cell implantation (when tumors were palpable) and continued until day 20 with a 1-day resting period every 5 days. TW-37 was resuspended in 1:1 Tween 80/ethanol (diluted 10-fold in 0.9% saline before use) and given daily at 40 mg/kg alternating i.v. or i.p. injections. CI-1040 was treated cells as a reference.

**Statistics.** Statistical analyses of drug response in mouse xenograft models were done using the SAS statistical software (SAS Institute, Inc. Cary, NC). The Tukey’s HSD test was used for pairwise comparisons among groups, and the Dunnett test for individual comparisons to untreated controls. The type I error rate was set at 0.05.

**Results**

**Identification of melanoma cell lines resistant to inhibition of the MAPK pathway.** It has been recently reported that NRAS- and BRAF-expressing melanoma cells have a different sensitivity to inhibitors of the MAPK pathway (25). Thus, metastatic melanoma cells with NRAS mutations have an increased resistance to RAF and MEK inhibitors (25). To identify poorly responsive cells and address the molecular basis underlying the resistance to MAPK inhibition, a panel of 11 melanoma cell lines was sequenced for the most frequent mutational hotspots in the NRAS and BRAF genes: exon 3 in NRAS and exon 15 in BRAF (see Fig. 1A and B). The individual cell lines were subsequently compared in their response to the MEK inhibitor U0126 (42), which blocks ERK activation downstream of NRAS or BRAF (Fig. 1C). U0126 was able to inhibit cell proliferation (Fig. 1A) by a G1-S mediated cell cycle arrest in NRAS- and BRAF-mutated cells (Fig. 1D). However, as a death inducer, U0126 is poorly effective; thus, at concentrations required to maintain the viability of normal melanocytes, the NRAS-mutated cells and three of five BRAF\(^{V\text{600E}}\)-expressing melanoma lines responded poorly to U0126 (Fig. 1B). In fact, the overall killing activity by this MEK inhibitor was not significantly different from standard chemotherapeutic drugs, such as Adriamycin (Fig. 1B). Two of the most resistant lines (SK-Mel-103 and SK-Mel-147) were chosen as representative examples to identify survival mechanisms acting in the absence of ERK activation and to test new compounds able to overcome melanoma chemoresistance.

**Antiprotective factors retained after ERK inhibition.** Despite the ability of U0126 to block ERK phosphorylation, it was conceivable that downstream apoptotic targets (i.e., Bim\(^{EL}\), Bcl-X\(_L\), Bcl-2, or Mcl-1) were not affected by treatment. To address this possibility, protein extracts were prepared from melanoma cells at different points after incubation with U0126. As shown in Fig. 1E, although Bim\(^{EL}\) was induced by U0126, Bcl-2 and Bcl-X\(_L\) were still detectable at late times after treatment, and Mcl-1 levels did not significantly change (Fig. 1E). With respect to other apoptotic factors frequently associated with melanoma chemoresistance, it was intriguing that the levels of SURVIVIN were nearly abrogated by U0126, but no significant cell death was observed (Fig. 1E). Therefore, in contrast to other cell types (20-22, 43, 44), Mcl-1 (as well as Bcl-2 and Bcl-X\(_L\)) is largely independent of MEK/ERK. Furthermore, up-regulation of Bim\(^{EL}\) and inhibition of SURVIVIN are not sufficient per se to promote cell death in aggressive melanoma cells.

**Stable RNA interference for target validation.** To determine the relative contribution of Mcl-1, Bcl-2, and Bcl-X\(_L\) to U0126-mediated resistance, a lentiviral-mediated approach was used to stably express specific shRNAs in melanoma cells. To allow for imaging of infected cells, the KH1-LV lentivirus was used, which coexpresses enhanced GFP under an independent UbC promoter (Supplementary Fig. S1A and B). Constructs able to promote a reduction of >80% of the intended protein expression without affecting the level of other Bcl-2 family members are shown in Supplementary Fig. S1C.
Acute inactivation of Bcl-2, Bcl-xL, or Mcl-1 by shRNA significantly enhanced the response of melanoma cells to U0126 (Fig. 2A). Interestingly, the most effective cytotoxic effect was found after inactivating Mcl-1 (Fig. 2A), consistent with this protein remaining highly expressed in melanoma cells after treatment with U0126 (Fig. 1E).

Altogether, these results indicate that despite the multiple genetic defects that melanoma cells harbor, the resistance to MEK/ERK inhibition is primarily dependent on Mcl-1 and to a lesser extent on Bcl-xL and Bcl-2.

Pharmacologic enhancement of the response of melanoma cells to U0126: design and validation of new BH3 mimetics. Small molecule inhibitors that interfere with antiapoptotic members of the Bcl-2 family are emerging as a powerful anticancer strategy (45). Nevertheless, published synthetic BH3 mimetics either do not recognize or bind poorly to Mcl-1 (46–48). Therefore, we used a structure-based methodology to generate novel non-peptide small molecules able to bind Mcl-1 as well as Bcl-2 and Bcl-xL. Our approach was based on the reported ability of the BH3 domain of the proapoptotic Bim protein to bind in a promiscuous manner to Mcl-1, Bcl-xL, and Bcl-2 (45, 49). Using the structure of Bim for computational docking and molecular dynamics, a series of putative BH3 mimetics were designed, of which the compound TW-37 (Fig. 2F) was chosen for displaying a high cell permeability (data not shown).

Fluorescence polarization–based competitive binding assays were done to address the ability of TW-37 to displace short peptides containing the BH3 domain of Bim or Bid from Bcl-2, Bcl-xL, or Mcl-1. Representative examples of binding curves are shown in Fig. 2C. TW-37 was found to bind Mcl-1, Bcl-xL, and Bcl-2 with Ki of 260, 1,100, and 120 nmol/L, respectively (Fig. 2C; results not shown).

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Selective and synergistic killing of melanoma cells by U0126 and TW-37. Aggressive melanoma lines, such as SK-Mel-103 and SK-Mel-147, could be killed with TW-37 at concentrations of 10 μmol/L (data not shown). Interestingly, lower drug concentrations, although inducing minimal toxicity, were found to be highly synergistic with U0126 (see isobolograms at EC50 and EC80 in Fig. 2D; representative microphotographs of treated cells are shown in Fig. 2F). Confirming the BH3-binding features of TW-37, the inactive TW-37i was unable to synergize with U0126 (Fig. 2E).

Notably, the U0126/TW-37 combination was well tolerated by melanocytes in short-term treatments (Fig. 2B). Therefore, TW-37 was used as an inactive control.
further illustrating the selectivity of this drug combination towards tumor cells (Supplementary Fig. S2B).

Importantly, in melanoma cells, the combination of TW-37/U0126 induced hallmarks of apoptosis, including a synergistic processing of regulatory and effector caspases (Supplementary Fig. S3A) as well as classic chromatin condensation and formation of apoptotic bodies (Supplementary Fig. S4). It should be noted, however, that an important fraction of cells could still die in the presence of the pan-caspase inhibitor zVAD-fmk (Supplementary Fig. S3B). This feature of the TW-37/U0126 combination may be advantageous to kill melanoma cells even under conditions of defective caspase activation, which has been proposed as a main contributor to the resistance to standard chemotherapeutic agents (6, 8).

Mechanistic analyses of the TW-37/U0126 combination: release of proapoptotic factors from the mitochondria. The enhanced activity of TW-37 in the presence of U0126 prompted us to address the interplay between BH3-containing proteins and the MAPK pathway. An attractive feature of BH3 mimetics as anticancer agents is their potential ability to promote cell death by favoring the release of cytochrome c and other mitochondrial death inducers by directly activating BAX and BAK (49).

As shown in Fig. 3A, low doses of TW-37 allowed for the release of cytochrome c, Smac, and AIF from the mitochondria (consistent with the function of TW-37 as a BH3 mimetic; ref. 45). Interestingly, U0126 greatly accelerated the effect of TW-37 on the mitochondria, shifting the detection of cytosolic cytochrome c by immunoblotting from 40 hours to as early as 6 hours posttreatment (Fig. 3A; see also Fig. 3B and Supplementary Fig. S4 for representative analysis of the visualization of cytochrome c release by immunofluorescence). Subsequent activation of BAX/BAK was important for TW-37/U0126-mediated cell death because down-modulation of these apoptotic factors favored melanoma cell survival. Thus, shRNA-expressing lentiviruses were generated to block BAX or BAK

**Figure 2.** TW-37 as a novel class of BH3 mimetics. Tumor cell–selective enhancement of the cytotoxic effect of U0126. A, genetic inactivation of Bcl-2, Bcl-xL, or Mcl-1 by RNA interference synergizes with U0126. Death responses of the indicated melanoma cell populations in the absence (white columns) or presence (gray columns) of U0126 (10 μmol/L, 48 hours). Cell death was assayed in triplicate by trypan blue exclusion. Extent of cell death relative to shRNA scramble control-infected cells. B, molecular structure of the small molecule inhibitor TW-37 and the inactive TW-37i derivative. C, binding kinetics of TW-37 to the antiapoptotic Bcl-2, Bcl-xL, and Mcl-1 proteins estimated by fluorescence polarization-based spectroscopy (see Materials and Methods). D, isobolograms for a graphical visualization of the synergistic effect of the TW-37/U0126 combination. EC<sub>50</sub> (top) or EC<sub>80</sub> (bottom) obtained from indicated drug given as a single agent and tested in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The data points corresponding to combination treatments fall below the line of additivity (broken line), indicating a supra-additive (synergistic) interaction between TW-37 and U0126. E, cytotoxicity of TW-37 or the inactive TW-37i variant (5 μmol/L each) in the absence or presence of U0126 (10 μmol/L). Microphotographs of the indicated melanoma cell lines or normal control melanocytes 40 hours after treatment. Note the preferential toxicity of TW-37/U0126 towards the tumor cells (see Supplementary Fig. S2 for long-term effects of the different treatments).
expression by RNA interference (Fig. 3 C and D). shRNA of BAX reduced by 50% the killing activity of TW-37/U0126 in line SK-Mel-103 (Fig. 3 E, left). SK-Mel-147 required BAX and BAK for full induction of cell death because shRNA against each of these proteins reduced TW-37/U0126–driven cell killing (Fig. 3 E, right).

Role of MEK/ERK inhibition upstream of BAX. BRAF and ERK have been reported to act downstream of cytochrome c or Smac to control caspase activation (19, 23). However, the synergistic effect of U0126 on cytochrome c release suggests an additional role of the MAPK upstream of the mitochondria, controlling BAX/BAK activation. To this end, we used antibodies that can specifically recognize conformational changes associated with pro-apoptotic activation of BAX by immunofluorescence staining (39, 40). We specifically focused on BAX as it contributed to the death of both SK-Mel-103 and SK-Mel-147. Interestingly, at the dose and treatment regimen in this study, no significant activation of BAX by TW-37 was detected unless in the presence of U0126 (Fig. 4 A). Thus, TW-37/U0126 increased by 7- and 10-fold the percentage of cells with conformationally active BAX in SK-Mel-147 and SK-Mel-103, respectively (see Supplementary Fig. S5 for time course analyses). These results suggest a role for MEK/ERK in the control of BAX and the mitochondrial pathway in melanoma cells.

ROS modulating the cytotoxic effect of TW-37/U0126. Dysregulation of cellular redox mechanisms can be potent activators of caspase-dependent and caspase-independent forms of cell death (50). Therefore, we tested the possibility that MEK inhibition was cooperating with TW-37 in allowing for ROS accumulation. Towards this end, drug treatments were done in the presence of potent antioxidants. Two structurally unrelated antioxidants were used: Tiron, a spin trap, and Trolox, a water-soluble vitamin E analogue (32). When added simultaneously with TW-37, both of these antioxidants blocked TW-37/U0126 drug synergy, preventing BAX activation (Fig. 4 A) and significantly

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**Figure 3.** Classical BH3-mimetic features of TW-37. BAX/BAK-dependent activation of the mitochondrial apoptotic pathway. A, time course illustrating the release of mitochondrial apoptotic effectors cytochrome c (Cyt c), Smac, and AIF in response to the indicated treatments. Representative SDS-PAGE gels of cytosolic extracts isolated by digitonin fractionation. B, visualization of cytochrome c release from the mitochondria (white arrows) by immunofluorescence. C and D, requirement of BAX (C) and BAK (D) for TW-37/U0126–driven melanoma cell death. Immunoblots illustrating the efficacy and selectivity of the shRNA-lentiviral approach used (see Materials and Methods for additional information about the sequences targeted). E, the amount of cell death (%) was determined by trypan blue exclusion assay 40 hours after drug treatment. Columns, mean of three independent experiments; bars, SE.
reducing the kinetics and extent of cell death by TW-37/U0126 (Fig. 4B; results not shown). Note that the inhibition of cell death by Tiron or Trolox was stronger than the blockage of caspases by zVAD (Supplementary Fig. S3) or BAX and BAK RNA interference, suggesting a key role of ROS in TW-37/U0126–mediated cell death. The protective effect of Tiron or Trolox was compromised if they were added 12 hours after treatment (data not shown), suggesting an early contribution of ROS to melanoma cell death by TW-37/U0126. Production of ROS by TW-37 and further enhancement by U0126 were visualized in cultured cells with the oxidation-sensitive fluorescent probe CM-H2DCFDA (see Fig. 6C). These data are intriguing because they implicate a MAPK-dependent control of ROS production that cooperates with antiapoptotic Bcl-2 family proteins in the maintenance of melanoma cell viability.

**ROS-dependent activation of p53 by TW-37/U0126.** ROS are notorious for the pleiotropic effects that they can elicit in mammalian cells. To identify direct mediators of ROS-driven cell death among a plethora of byproducts of changes in cellular redox, we focused on proapoptotic factors whose expression is induced at early time points after TW-37/U0126 treatment but can be blocked by antioxidants. A protein that followed this expression pattern was p53. As shown in Fig. 4C and D, TW-37 was able to induce sustained expression of p53 in SK-Mel-103 and SK-Mel-147. Interestingly, the addition of U-0126 to TW-37 enhanced 12- to 15-fold the induction of p53 (Fig. 4C and D). This up-regulation of p53 was reduced by 80% in the presence of Trolox (Fig. 4C and D). Therefore, our results are consistent with the BH3 mimetic TW-37 and the MEK inhibitor U0126 activating p53 via ROS production.
To confirm the requirement of p53 for TW-37/U0126–mediated melanoma cell death, p53 protein expression was down-modulated by highly effective lentiviral vectors (Fig. 5A). Interestingly, p53 knockdown provided a protection from melanoma cell death by about 75% (Fig. 5B) and significantly reduced the activation and translocation of BAX by TW-37/U0126 (Fig. 5C). This is in contrast to standard chemotherapeutic agents, such as Adriamycin, etoposide, or cisplatin, which can induce p53 but cannot effectively engage the apoptotic machinery in aggressive melanoma cells (6, 8, 33).

**p53 and ROS define the tumor cell–selective toxicity of TW-37/U0126.** As melanocytes do not die in response to TW-37/U0126 (Fig. 2E), a corollary of our results is that the activation of the ROS/p53 apoptotic loop is restricted to tumor cells. To evaluate this possibility, normal melanocytes were compared in their response to melanoma cells. Although a significant accumulation and activation (by phosphorylation) of p53 could be detected in melanoma cells, normal melanocytes remained unaffected by TW-37, U0126, or the combination of both agents (Fig. 6A and B). Furthermore, the redox indicator CM-H2DCFDA revealed a striking difference in the production of ROS by melanoma cells and normal melanocytes. Thus, melanocytes remained negative for the production of oxidized DCF-dependent fluorescence even at late times posttreatment with TW-37/U0126 (Fig. 6C; results not shown). Yet, melanocytes could respond to strong ROS inducers, such as H2O2 (data not shown). With respect to mock-treated controls, melanoma cells incubated with TW-37 showed a 3-fold increase in the DCF-dependent signal, which was doubled in combination with U0126 (Fig. 6C, right).

To further validate the differential ability of melanocytes and melanoma cells to produce and respond to ROS induction, global expression of oxidized proteins was monitored by protein immunoblotting. Specifically, the presence of carbonyl groups (generated by oxygen-free radicals and other reactive species) was visualized after derivatization reactions with DNPH and staining with anti-DNP antibodies. Interestingly, the basal levels of DNPH-dependent staining were found to be already higher in untreated melanoma cells than in melanocytes (Fig. 6D). Treatment of melanoma cells with TW-37, but not the inactive TW-37i, led to a noticeable increase in oxidized proteins that was further exacerbated by U0126 (Fig. 6D). Importantly, no such changes were observed in normal melanocytes. Together, our results identify a new BH3 mimic (TW-37) as a novel strategy to exploit the differential redox metabolism of melanocytes and melanoma cells and subsequent activation of p53-mediated death programs.

**General cooperation between MEK inhibitors and TW-37: anticancer activity in vivo.** U0126 has been broadly used as a MEK inhibitor. However, to rule out putative unspecific effects of this...
compound, additional viability studies were done with CI-1040, a structurally different MEK inhibitor (51). As with U0126, CI-1040 was able to promote a tumor cell–selective killing of melanoma cells in the presence of TW-37 (Fig. 7A and B). Thus, CI-1040 enhanced by 5-fold the death of TW-37-treated melanoma cells without affecting the viability of normal melanocytes (Fig. 7A). Moreover, confirming the results with U0126, the synergistic effect of CI-1040 and TW-37 was strictly dependent on the production of ROS. Thus, both Tiron and Trolox completely blocked the cytotoxic activity of the TW-37/CI-1040 combination in melanoma cells (Fig. 7B). CI-1040 has been previously used as the proof of principle for blocking MEK in human melanoma cells grown as mouse xenografts (41). Therefore, we used this compound to validate our hypothesis that BH3 mimetics targeting Mcl-1, Bcl-xL, and Bcl-2 can significantly improve the therapeutic effect of MEK inhibition in vivo, even in otherwise chemoresistant melanoma cells expressing NRAS mutations. Towards this end, SK-Mel-147 were transduced with GFP (to aid in noninvasive imaging of tumor growth) and injected s.c. in immunosuppressed mice (Fig. 7C–F). Animals were treated with suboptimal concentrations of TW-37 and/or CI-1040 (to mimic unfavorable situations that may be encountered in human tumors) and monitored for tumor growth at different times after implantation. Consistent with the synergistic tumor cell killing in culture, the MEK inhibitor/TW-37 combination was found to block melanoma cell growth in mice as shown by a significant reduction in tumor volume and tumor mass (Fig. 7C and D; see representative examples of placebo and drug-treated animals in Fig. 7E and F and quantification of the reduction in tumor mass in Fig. 7D). In summary, our results identify a new BH3 mimetic (i.e., TW-37) as a potent strategy to overcome melanoma chemoresistance. Thus, rationally designed BH3 mimetics may broaden the spectrum of patients that could benefit from available inhibitors of the MAPK pathway.

**Discussion**

Here, we describe an unexpected interplay between the MAPK pathway and antiapoptotic factors in the control of melanoma cell viability. In addition, we report a new strategy to activate the intrinsic expression of p53 in melanoma cells by exploiting their endogenous sensitivity to ROS. This study capitalizes on stable RNA interference to define the specific role of single proteins in the
intrinsically complex genetic background of tumor cells. By combining pharmacologic approaches with selective down-modulation of Bcl-2, Bcl-x<sub>L</sub>, Mcl-1, BAX, BAK, and/or p53, we were able to (a) identify mechanisms of resistance to MEK inhibitors, (b) provide the rationale for a pleiotropic BH3 mimic (TW-37), (c) address the mode of action of this compound, and (d) define a differential regulation of ROS production in melanocytes and melanoma cells.

Dissecting the molecular basis underlying the regulation of the MAPK pathway and Bcl-2 family members has important translational implications. The potentially "druggable" nature of both signaling cascades (15, 16, 45) and the fact that they are invariably dysregulated in melanoma cells have inspired efforts aimed at the development of molecularly targeted therapies. Unfortunately, improved antitumor efficacy and reduced secondary toxicity from novel therapies has not yet been shown in clinical settings (28). Certainly, multiple factors may contribute to marginal effects of current therapeutic agents. Limited solubility and stability of the compound(s) as well as increased drug efflux pumps or detoxification enzymes are some examples of factors that may compromise the bioavailability of anticancer drugs in melanoma cells (6, 8). Our results support the notion that melanoma cells may be more resistant than other tumor cells by virtue of diversifying the regulation of death mediators, for example by reducing the number of antiapoptotic proteins controlled by the same transcription factor. Thus, ERK-independent expression of Mcl-1, Bcl-x<sub>L</sub>, and Bcl-2 (Fig. 1E) can provide a potent fail-safe mechanism for the maintenance of melanoma cell viability after RAS, BRAF, or MEK inhibition. Conversely, ERK-dependent down-regulation of apoptotic activators of BAX/BAK (e.g., BimEL) and the expression of survivin can prevent the induction of cell death by BH3 mimetics.

In the context of mechanistic analyses of cell death, TW-37 also sheds light on the requirements for the activation of the apoptotic

Figure 7. Synergy between TW-37 and MEK inhibitors is not restricted to U0126 and can be visualized in vivo. A, comparative analysis of the response of melanocytes (left) and the melanoma cell line SK-Mel-103 (right) to TW-37 in the absence or presence of the MEK inhibitor CI-1040. Columns, mean at 20 and 40 hours posttreatment (white and gray columns, respectively); bars, SE. Note the preferential killing of melanoma cells (but not melanocytes) by TW-37/CI-1040. B, inhibition of the synergistic effect of TW-37/CI-1040 by treatment with the antioxidants Tiron and Trolox, supporting a critical role of ROS in the killing of melanoma cells by the combination of BH3 mimetics and MEK inhibitors. C to F, effect of TW-37 and MEK inhibition on melanoma growth (mouse xenografts). C, comparative analysis of the localized growth of melanoma cells (line SK-Mel-147) implanted s.c. in immunosuppressed mice. Points, mean tumor volume in animals treated with placebo control (gray diamonds), TW-37 (gray squares), the MEK inhibitor CI-1040 (black triangles), or a combination of both agents (white squares); bars, SE. Pairwise comparisons among groups were analyzed with the Tukey's HSD test. *, P < 0.05, statistical significance with respect to untreated controls or the indicated drugs as single agents. D, tumor weight estimated for the indicated treatment groups at day 20 after implantation. The Dunnett test was used for individual comparisons to untreated controls. *, P < 0.05. E, representative photographs captured with visible light of animals corresponding to each treatment group at day 20 after tumor cell injection. F, noninvasive visualization of GFP-tagged tumor cells by whole-body fluorescence imaging showing a significant reduction in tumor size by the TW-37/MEK inhibition combination.
machinery in melanoma cells. The molecular basis of the resistance to standard chemotherapeutic agents remains unclear (6, 8, 28). Extrapolating from other tumor types has been complicated because of a debated controversy on the hierarchical organization of Bcl-2 family members. Specifically, a major point of contention has revolved around the activation of BAX and BAK (49, 52). Two main models have been described depending on how BAX and BAK become activated once they are released from antiapoptotic Bcl-2 members. According to the so-called “displacement model,” the default state of BAX and/or BAK is an active conformation able to directly cause release of proapoptotic proteins from the mitochondria (53, 54). In this setting, BH3 mimetics are expected to be highly effective because they would bypass the requirement for additional upstream activators of the mitochondrial pathways (i.e., Bim, Bid, or p53), which are frequently compromised in tumor cells.

The “direct binding model” argues that removal of antiapoptotic proteins is not sufficient to promote cell death, and that additional proapoptotic inducers are required for full activation of BAX and BAK (52, 55). Our data are consistent with this second model because low doses of TW-37 or acute inactivation of Bcl-2, Bcl-xL, or Mcl-1 by RNA interference were unable per se to engage the apoptotic machinery in melanoma cells. These results may account, at least in part, for the failure of Bcl-2 antisense strategies as monotherapy in melanoma (56). Taken at face value, our results would not even support the use of pleiotropic BH3 mimetics as single anti-melanoma agents. However, it should be emphasized that the very need for cooperative signals provides the basis for tumor cell selectivity. We propose MEK/ERK inhibition as an effective strategy to accelerate the kinetics and efficacy of BH3 mimetics. Thus, the induction of BimL and reduction of survivin by U0126, together with the synergistic effect of U0126 and TW-37 on p53, could provide the required signals for the activation of BAX/BAK and the subsequent induction of cell death in otherwise chemoresistant melanoma cells.

Perhaps one of the most intriguing results of this study is that the synergy between TW-37 and the inactivation of MEK/ERK relies on a tumor cell–restricted induction of p53 via ROS. Functional interactions between p53 and MAPK pathways have been described in a variety of systems. Thus, the MAP kinases, ERK, c-Jun NH2-terminal kinase, and p38 can play an active role in the phosphorylation and induction of p53 (57). However, in melanoma cells treated with a BH3 mimic, we found the opposite situation: inhibition of MEK/ERK favored an efficient accumulation and activation of p53. Future studies will determine the specific effect of ROS on p53 function, but it may correspond to direct activation by oxidation (58).

Importantly, the TW-37/U0126 combination offers several advantages. First, the induction of p53 by TW-37/U0126 is tumor cell selective. This is in contrast to stimuli such as UV and γ-radiation and various DNA-damaging drugs, including Adriamycin, etoposide, or cisplatin among others, which affect p53 levels and frequently compromise in normal cell compartments, TW-37/U0126 could reduce the secondary toxicity characteristic of standard antitumor therapies. A second attractive feature of TW-37/U0126 is that it can exploit transcription-independent functions of p53 and thus bypass defects required for DNA binding (52). Thus, BAX and BAK activation were observed independently of significant increases in total protein expression (Fig. 4C and D).

Furthermore, TW-37/U0126 can efficiently bypass defects downstream of the mitochondria. Of note, the melanoma lines used in this study express low levels of APAF-1 and high levels of caspase inhibitors (e.g., XIAP and ML-IAP; refs. 32, 59). These genetic defects, which can reduce the sensitivity to Adriamycin, paclitaxel, or high doses of etoposide (59–62), did not prevent cell death by TW-37/U0126.

Finally, the TW-37/U0126 treatment revealed an intrinsically different threshold for the accumulation and control of changes in ROS between normal melanocytes and melanoma cells. Melanocytes are specialized pigment-producing cells. They produce melanin, which is inherently adapted to scavenge ROS and thus prevent DNA damage, recruitment of stress-associated transcription factors, and the initiation of apoptosis (63). Paradoxically, this protective function of melanin is frequently lost during tumor progression (64). Consequently, melanoma cells may be more sensitive than melanocytes to ROS-induced cell death (65). The accumulation of ROS following the addition of U0126 in melanoma cells treated with TW-37 indicates that the MEK/ERK MAPK pathway may play an additional role in controlling the mechanism of melanoma viability under ROS-inducing stress stimuli.

In conclusion, here, we have shown a potential therapy for melanoma based on the capacity of a novel, pleiotropic BH3 mimetic (TW-37) to synergize with MEK inhibition (U0126). We have shown that melanoma cell death is dependent not only on the activation of BAX/BAK as expected from a BH3 mimetic, but a tumor cell–selective induction of a ROS/p53 feedback loop upstream of the mitochondria. Therefore, this combination therapy may prove especially beneficial for melanoma because p53 is rarely mutated in this tumor type (6). The TW-37/U0126 combination takes full advantage of intrinsic dysregulated redox capacity of melanoma cells and highlights ROS as a point of vulnerability of melanoma cells that can be exploited for drug development.

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