Deciphering the Molecular Events Necessary for Synergistic Tumor Cell Apoptosis Mediated by the Histone Deacetylase Inhibitor Vorinostat and the BH3 Mimetic ABT-737

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Abstract

The concept of personalized anticancer therapy is based on the use of targeted therapeutics through in-depth knowledge of the molecular mechanisms of action of these agents when used alone and in combination. We have identified the apoptotic proteins and pathways necessary for synergistic tumor cell apoptosis and in vivo antitumor responses seen when the HDAC inhibitor vorinostat is combined with the BH3-mimetic ABT-737 in lymphomas overexpressing Bcl-2. Vorinostat “primes” tumors overexpressing Bcl-2 for rapid ABT-737–mediated apoptosis by inducing expression of the BH3-only gene bmf. Moreover, these synergistic effects of vorinostat/ABT-737 were blunted in cells with an inactive p53 pathway or in cells lacking expression of the p53 target gene, noxa. These studies show the important and complex functional interaction between specific proapoptotic BH3-only proteins and the BH3-mimetic compound ABT-737 and provide the most comprehensive functional link between tumor genotype and the apoptotic and therapeutic effects of HDACi combined with ABT-737. Cancer Res; 71(10); 3603–15. ©2011 AACR.

Introduction

Histone deacetylase inhibitors (HDACi) can induce tumor cell apoptosis, inhibit cell proliferation by blocking progression through the G1/S or G2/M cell cycle checkpoints, induce cellular differentiation, suppress angiogenesis, and modulate tumor immunity (1). Using genetic mouse models of cancer, we and others have shown that direct tumor cell killing by these agents is essential for their antitumor responses in vivo (2, 3). Some studies have indicated that apoptosis mediated by HDACi proceeds through the extrinsic (death receptor) pathway following transcriptional upregulation of tumor necrosis factor superfamily ligands and/or receptors (2, 4–7). However, in the majority of cases including all studies that we have performed, activation of the intrinsic (mitochondrial) apoptosis pathway plays a fundamental role in mediating HDACi-induced cell death (3, 8–12).

The intrinsic apoptosis pathway is regulated by pro- and antiapoptotic Bcl-2 family proteins that consists of 3 major subgroups: (i) multidomain prosurvival proteins (Bcl-2, Bcl-xL, Bcl-w, McI-1, A1); (ii) BH3-only proapoptotic proteins (Bid, Bim, Bik, Bmf, Noxa, Puma, Hrk, Bad); and (iii) multidomain proapoptotic proteins (Bax, Bak, Bok; ref. 13). ABT-737 is a BH3 mimetic compound developed to specifically inhibit the activity of prosurvival Bcl-2 family proteins (14). Using competitive fluorescence polarization assays and recombinant Bcl-2 family proteins, ABT-737 was shown to bind with relatively high affinity to Bcl-2, Bcl-xL, and Bcl-w and to have much lower capacity to bind to and inhibit McI-1 and A1 (14). Our subsequent cell-based assays confirmed that ABT-737 was a potent inhibitor of Bcl-2 and Bcl-xL, and a poor inhibitor of McI-1 and A1, but surprisingly was only a weak inhibitor of overexpressed Bcl-w in Eu-ncy lymphoma cells (15).

Although HDACi show great promise as cancer therapeutics, given their diverse molecular and biological antitumor activities and their manageable clinical side effects, these agents may be best utilized in the clinic in combination with existing chemotherapeutics or with novel small molecule or biological agents in a targeted manner (16). We previously showed that overexpression of prosurvival Bcl-2 proteins inhibits the apoptotic and therapeutic activities of HDACi (3, 17) and combination treatment with ABT-737 restored the efficacy of HDACi against tumors overexpressing Bcl-2 and Bcl-xL (15). Although these studies clearly established that combining HDACi and ABT-737 resulted in more potent antitumor activities in vitro and in vivo, the molecular events...
necessary for synergistic tumor cell apoptosis mediated by the combination treatment had not been elucidated.

Herein we show that the HDACi, vorinostat, “primed” tumor cells overexpressing Bcl-2 for rapid apoptosis following treatment with ABT-737 by inducing expression of certain BH3-only genes. We determined that upregulation of bmf, but not bim, by vorinostat was functionally critical to prime cells for apoptosis mediated by ABT-737 in vitro and in vivo. Highly expressed Bmf was bound by Bcl-2 at baseline, however, the addition of ABT-737, which avidly binds to Bcl-2, resulted in release of Bmf from Bcl-2, allowing subsequent interaction between Bmf and Mcl-1. Synergistic apoptosis induced by vorinostat and ABT-737 was suppressed in cells with mutated p53, although the induction of BH3-only genes by vorinostat in the absence of a functional p53 pathway was unaffected. The functional importance of Bmf and p53 in mediating a robust response to vorinostat and ABT-737 was shown in cells with knockout or knockdown of p53, although the induction of BH3-only genes by vorinostat in the absence of a functional p53 pathway was unaffected. The functional importance of Bmf and p53 in mediating a robust response to vorinostat and ABT-737 was shown in cells with knockout or knockdown of bmf and mutatd p53 that displayed a severely attenuated response to the vorinostat/ABT-737 combination. We showed that basal expression of the p53 target gene, noxa, and to a lesser extent, puma, was reduced in cells with mutant p53. Finally, knockout of noxa in p53-competent, Bcl-2-overexpressing cells also suppressed the apoptotic response to the combination of vorinostat and ABT-737.

These studies provide important mechanistic insight into the molecular events that underpin synergistic tumor cell death mediated by HDACi combined with ABT-737. Bmf was a common transcriptional target of structurally diverse HDACi and induction of this gene was critical for the combined effects of vorinostat and ABT-737. This defines a novel functional relationship between Bmf and ABT-737 in killing cells overexpressing Bcl-2 and highlights the concept of "apoptotic priming" of tumor cells by an anticancer agent for death mediated by a second agent. In this instance, vorinostat-induced induction of bmf "primed" tumor cells overexpressing Bcl-2, for subsequent death mediated by ABT-737. However, the cells were additionally sensitized to ABT-737-mediated apoptosis through the expression of wild-type p53, likely through the maintenance of adequate basal levels of noxa and puma.

Materials and Methods

Drugs and compounds

Vorinostat (Merck), panobinostat (Novartis), romidepsin (Gloucester), VPA (Sigma-Aldrich), etoposide, taxol, vincristine (Peter MacCallum Cancer Centre), ABT-737 (Abbott Park), were dissolved in dimethyl sulfoxide (DMSO). For in vivo experiments vorinostat and ABT-737 were prepared as previously described (15).

Cell culture and Western blotting

Development of Eμ-myc, Eμ-myc/bcl-2, Eμ-myc/mcl-1, Eμ-myc/bim⁻/⁻, Eμ-myc/bmf⁻/⁻, Eμ-myc/noxa⁻/⁻, Eμ-myc/apaf-1⁻/⁻, and Eμ-myc/p53⁻/⁻ lymphomas has been previously described (3, 15, 18–20). MSCV retroviral plasmids for expression of Bcl-2 and A1 were described previously (15). For gene knockdown studies, shRNA mirs in the pLMP vector directed against p53 (kindly provided by Dr Ross Dickens, Walter and Eliza Hall Institute), bmfshRNA1 (sense—AGCAAGCCAGGCTGAACCTAA), bmfshRNA2 (sense—CGGCGAGGATGAGAATCTT), and I1F1 16 (sense—CTCATATCGATTATTTTGGAA) were used. Retroviral transduction of Eμ-myc lymphoma cells and Western blotting was performed as previously described (3). Blots were probed with α-Bcl-2 (BD Sydney), α-Bim (Stressgen), α-Bmf (Alexis), α-p53 (Novocastra), α-acetylated-Histone H3 (Upstate), and α-ß-actin (Sigma) used as a control. Lymphomas were also analyzed for p53 pathway status by treatment with 20 nmol/L etoposide Western blotting with α-p53 (Novocastra) and α-p19ARF Invitrogen and cell death as measured by propidium iodide (Sigma-Aldrich) uptake and annexin V-APC (BD Biosciences) binding.

Synergistic cell death and colony formation

Eμ-myc/bcl-2 (1 × 10⁶ cells) were incubated for 8, 16 hours with 2.5 μmol/L vorinostat then washed with media and incubated and for a further 1, 2, 4, and 8 hours with 0.1 μmol/L ABT-737 or 0.1 μmol/L ABT-737e (an enantiomer of ABT-737) or 4 and 8 hours with ABT-737 or ABT-737 treatment alone. Eμ-myc lymphoma cells (1 × 10⁴ cells/mL) were also incubated in the presence of the indicated compounds for 24 hours. Cell death was analyzed by propidium iodide (Sigma-Aldrich) uptake and annexin V-APC (BD Biosciences) as described (3, 15). Colony assays were performed as previously described (17). Synergy was calculated using the Biosoft CalcuSyn program based on the Chou-Talalay method (21).

Immunoprecipitation assays

For immunoprecipitation, Bcl-2 and Mcl-1 antibodies were bound to Protein G Sepharose 4 Fast Flow-beads (Amersham) and cross-linked using 40 mmol/L dimethyl pimelidate in 0.1 M borate (pH 9). Eμ-myc/bcl-2 cells were treated with 0.5 μmol/L ABT-737 for 8 hours. Lysates were prepared on ice in lysis buffer (20 mmol/L Tris-HCl pH 7.4, 135 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L EDTA, 1% Triton X-100) supplemented with Complete protease inhibitors (Roche). Equilibrated antibody-bound beads were incubated with 50 μg protein at 4°C over night. After 4 washes with lysis buffer, the beads were boiled in SDS-sample buffer and analyzed by Western blotting.

RNA isolation, cDNA, and quantitative real time-PCR analysis

1 × 10⁶ cells were harvested in 250 μL of TRizol reagent (Invitrogen). RNA was isolated using an Invitrogen isolation method and cDNA synthesis was performed according to manufacturers instructions (Promega). Quantitative PCR analysis of samples was performed on ABI7900 light cycler with SYBER-green ROX mix (Thermo Scientific-Abgene) with primers spanning exons of the BH3-only family of proteins utilizing ribosomal housekeeping gene RPL-32. Primer sequences for the complete BH3 family set can be requested from the author.

In vivo apoptosis analysis and therapy

Approximately 5 × 10⁶ Eμ-myc/bcl-2 cells were injected intravenously (i.v.) into the tail veins of 5-week-old male mice.
C57BL/6 mice. Mice were monitored and treated when an average total white cell blood count of over $13 \times 10^6$ cells/mL as assessed by Bayer ADVIA 120 hematology analyzer (GMI Ramsey) showed leukemia. Therapy consisted of administration of vorinostat (200 mg/kg) for 12 hours and ABT-737 (100 mg/kg) for 4 hours later or each drug independently along with a 16-hour vehicle control.

Vorinostat and ABT-737 combination therapy was performed on $7 \times 10^4$ AT1 breast carcinoma cells were harvested and injected subcutaneously (s.c.) into the flanks of 4-week-old female Balb/c mice. Once the tumor reached 9 mm$^2$, daily simultaneous intraperitoneal (i.p.) administration of 200 mg/kg vorinostat and 150 mg/kg ABT-737 began. The mice were treated and monitored for 14 days. Eμ-myc/bcl-2 tumors from the lymph nodes of treated mice were subjected to immunohistological staining with hematoxylin/eosin and TUNEL analysis as previously described (17).

**Chromatin immunoprecipitation assay**

The chromatin immunoprecipitation (ChIP) assay was performed as per the manufacturer’s instructions using a cell signaling EZ ChIP kit (Upstate). Cross-linked protein/DNA was incubated with either 1 μg of isotype control antibody or 5 μg of antiacetylated histone H3 (Upstate) for 16 to 24 hours at 4°C. The immuno-precipitated DNA was analyzed by PCR with primers targeting the promoter regions of specific BH3-only genes (available from author).

**Results**

**Synergistic tumor cell apoptosis mediated by vorinostat and ABT-737**

Previously we showed that coincubation of HDACi and ABT-737 induced apoptosis in Bcl-2-overexpressing lymphomas derived from Eμ-myc transgenic mice (Eμ-myc/bcl-2; ref. 15). We hypothesized that HDACi may sensitize or “prime” cells for ABT-737–induced apoptosis. Treatment with vorinostat (2.5 μmol/L) alone for 24 hours had little or no effect on the viability of Eμ-Myc/bcl-2 lymphomas whereas treatment with ABT-737 (0.5 μmol/L) caused less than 20% of the lymphoma cells to undergo apoptosis (Supplementary Fig. S1A–D). However, pretreatment of Eμ-Myc/bcl-2 lymphomas with vorinostat (2.5 μmol/L) potently sensitized the tumor cells to ABT-737–induced apoptosis (Supplementary Fig. S1A–D). The synergistic effects of vorinostat and ABT-737 required effective inhibition of Bcl-2, as an enantiomer of ABT-737 with significantly less Bcl-2-inhibitory activity (14) did not synergize with vorinostat (Fig. 1A). We confirmed that a second independently derived Eμ-Myc/bcl-2 lymphoma was also sensitized to ABT-737–induced apoptosis following pretreatment with vorinostat (2.5 μmol/L) for 16 hours (Supplementary Fig. S1E).

**Vorinostat induces expression of a subset of proapoptotic BH3-only genes**

We hypothesized that vorinostat primed Eμ-Myc/bcl-2 lymphomas for ABT-737–mediated apoptosis by increasing the expression of proapoptotic BH3-only genes. Treatment of Eμ-myc/bcl-2 lymphomas with vorinostat alone or vorinostat and ABT-737 resulted in induction of 3 BH3-only genes, bmf, bim, and noxa, more than 2-fold above baseline (Fig. 1B and C). Interestingly, bmf (~12-fold), noxa, and bim (both ~3.5-fold) were induced to maximal levels following single agent treatment with vorinostat (Fig. 1B). In contrast, hrk was maximally induced (~100-fold) after 16 hours pretreatment with vorinostat followed by 8 hours incubation with ABT-737 (Fig. 1C). Only moderate induction of hrk (6-fold) was observed when Eμ-myc/bcl-2 lymphoma cells were treated with ABT-737 alone for 8 hours and none of the other Bcl-2 family genes showed consistent changes in expression following single agent ABT-737 treatment (Fig. 1B and C). Changes in the expression of Bmf, Bim, and Hrk protein mimicked the magnitude and kinetics of their transcriptional profiles (Fig 1D). All detectable Bmf isoforms showed increased expression following treatment with vorinostat (Fig. 1D, lanes 1–3), although there was a 1 hour delay in maximal protein expression compared to the kinetic change in bmf mRNA (compare with Fig. 1B). In contrast, there was no observable change in Bmf levels following treatment with ABT-737 alone (Fig. 1D). Similarly, the expression of BimL, BimS, and BimL protein isoforms increased over time in response to vorinostat in the presence and absence of ABT-737 (Fig. 1D). Densitometric analysis revealed that Bmf protein levels were induced maximally 11-fold above baseline, whereas BimL was induced 8-fold above baseline. Maximal levels of Hrk were observed following pretreatment with vorinostat for 16 hours and an additional incubation with ABT-737 for 4 to 8 hours (Fig. 1D, lanes 6 and 7). Changes in Noxa protein could not be evaluated by Western blotting, as a suitable antibody specific for endogenous mouse Noxa is not currently available. Expression of the BH3-only family member Bad and multidomain proapoptotic member Bax did not change during treatment with vorinostat and/or ABT-737 (Fig 1D). These results indicate that bmf, bim, and noxa are genes that are up-regulated during pretreatment with vorinostat and hence the protein products of these genes may “prime” Eμ-Myc/bcl-2 lymphoma cells for rapid apoptosis mediated by ABT-737.

To determine if bmf, bim, noxa, and hrk levels were altered following treatment with vorinostat in vivo, tumor and normal cells were harvested from the same lymph nodes of vorinostat-treated C57BL/6 mice bearing GFP Eμ-Myc lymphomas. Vorinostat induced a robust and early induction of bmf in Eμ-Myc lymphomas whereas little or no change was observed in normal cells (Supplementary Fig. S2A). More modest tumor cell-specific induction of bim and noxa was also observed following treatment of mice bearing Eμ-Myc lymphomas (Supplementary Fig. S2B and C). Maximal induction of hrk was observed at later time points (Supplementary Fig. S2D), consistent with the notion that transcriptional activation of this gene occurs as a consequence of apoptosis. Western blot analysis confirmed expression of Bmf in Eμ-myc/bcl-2 lymphomas isolated from the lymph nodes of vorinostat-treated tumor-bearing mice (Supplementary Fig. S2E).

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Vorinostat-induced upregulation of bmf, bim, and noxa occurs independently of apoptosis

To confirm that vorinostat-mediated upregulation of BH3-only genes was not a response to induction of apoptosis, Eμ-myc/bcl-2 cells were treated with vorinostat and ABT-737 in the presence of the pan-caspase inhibitor zVAD-fmk. Apoptosis mediated by vorinostat/ABT-737 was inhibited by zVAD-fmk (Supplementary Fig. S3A), however bmf, bim, and noxa mRNA still increased following treatment with vorinostat (Supplementary Fig. S3B). Of note, the induction of hrk following treatment with vorinostat and ABT-737 was suppressed compared to the level of gene induction seen in cells that underwent apoptosis following exposure to both agents (compare Figs. 1C and 3B). Similar results were obtained using Eμ-myc lymphomas overexpressing the prosurvival family member Bcl-2A1 (A1) that we had previously shown to be resistant to vorinostat/ABT-737 combination treatment (15), as ABT-737 does not inhibit A1 (refs. 22, 23; Supplementary Fig. S3C and D). Taken together these results suggest that vorinostat primes cells for ABT-737–mediated death by specifically inducing expression of certain BH3-only genes, namely bmf and to a lesser extent bim and noxa. In contrast, the upregulation of hrk following treatment with vorinostat and ABT-737 appears to be a secondary response triggered by apoptotic signaling and hrk is therefore unlikely to be important for vorinostat-mediated priming for death by ABT-737.

Vorinostat induces histone acetylation within the promoter region of BH3-only target genes

To determine if the transcriptional activation of selective BH3-only genes by vorinostat was due to drug-induced hyperacetylation of their promoter regions, ChIP assays were performed. Eμ-myc/bcl-2 cells were treated with vorinostat for 16 hours and ChIP assays were performed using an anti-acetylated-histone H3 antibody. Treatment with vorinostat...
resulted in very strong induction of histone H3 acetylation at the \( bmf \) promoter and weaker acetylation of histone H3 at the \( bim \) promoter (Supplementary Fig. S4A and B). This suggests that up-regulation of \( bmf \) and \( bim \) following treatment with vorinostat occurs as a direct consequence of changes in the levels of acetylation at the promoters of these target genes. Additional studies showed that vorinostat-mediated induction of \( bmf \), \( bim \), and \( noxa \) mRNA was not inhibited by continuous cotreatment of cells with cyclohexamide to inhibit de novo protein synthesis (data not shown). Together, these data indicate that vorinostat induces direct hyperacetylation of histone H3 within the promoter regions of genes such as \( bmf \) and \( bim \) resulting in their transcriptional upregulation.

**Vorinostat-mediated induction of \( bmf \) is important to sensitize cells for ABT-737-mediated apoptosis**

To determine the functional importance of Bmf and Bim for the synergistic killing of cells overexpressing Bcl-2 by vorinostat and ABT-737, we generated \( E\mu\)-myc/bcl-2 (19) lymphoma cells with overexpression of Bcl-2. Exposure of \( E\mu\)-myc/bcl-2, \( E\mu\)-myc/bim\(^{−/−}\)/bcl-2, and \( E\mu\)-myc/bmf\(^{−/−}\)/bcl-2 lymphomas to the vorinostat-ABT-737 combination revealed that \( E\mu\)-myc/bim\(^{−/−}\)/bcl-2 cells were as sensitive as \( E\mu\)-myc/bcl-2 cells whereas \( E\mu\)-myc/bmf\(^{−/−}\)/bcl-2 lymphomas displayed significantly less apoptosis (Fig. 2A). To confirm the important role of \( bmf \) in mediating the synergistic apoptotic effects of vorinostat and ABT-737, \( E\mu\)-myc/bcl-2 lymphomas were transduced with retroviral vectors expressing 2 different short hairpin RNAs (shRNA) directed toward \( bmf \) or an shRNA against the human \( IFI16 \) gene as a control. Both shRNAs targeting \( bmf \) robustly inhibited the expression of the proapoptotic Bmf1 protein isoform (Supplementary Fig. S5). Exposure of \( E\mu\)-myc/bcl-2 lymphomas expressing the \( IFI16 \) shRNA (\( E\mu\)-myc/bcl-2/\( IFI16\)shRNA) to vorinostat and ABT-737 resulted in apoptosis in these cells that was very similar in magnitude and kinetics to that observed in \( E\mu\)-myc/bcl-2 cells (Fig. 2B). In contrast, knockdown of \( bmf \) by 2 different shRNAs in \( E\mu\)-myc/bcl-2/\( bmf\)shRNA1 and \( E\mu\)-myc/bcl-2/\( bmf\)shRNA2 cells resulted in resistance to the combination treatment (Fig. 2B). These results confirm that Bmf plays an important role in mediating robust apoptosis of \( E\mu\)-myc/bcl-2 lymphomas upon treatment with vorinostat and ABT-737.

Given the important functional role of Bmf in priming vorinostat-treated cells for ABT-737–induced apoptosis, we wished to determine if other HDACi also specifically upregulated \( bmf \). Treatment of \( E\mu\)-myc/bcl-2 cells with the HDACi panobinostat (LBH589), valproic acid (VPA), romidepsin (depsipeptide), and vorinostat resulted in a robust increase in \( bmf \) mRNA that was substantially greater than the response of any other BH3-only gene assessed (Fig. 2C). In contrast, other apoptotic stimuli did not significantly induce \( bmf \).
(Fig. 2D). Consistent with previous reports (24, 25), treatment with the DNA damaging agent etoposide, exposure to UV irradiation, and deprivation of serum resulted in enhanced expression of the p53-response gene puma (Fig. 2D) whereas noxa was induced following etoposide treatment (Fig. 2D) as previously showed (26). Interestingly, treatment of Eμ-μc/myc-bcl-2 lymphomas with concentrations of taxol or vincristine capable of mediating ~50% death of Eμ-μc lymphomas (data not shown) did not cause any substantial increase in any of the BH3-only genes studied.

**Bmf is required for the antitumor responses to vorinostat and ABT-737 in vivo**

We next determined if the requirement for vorinostat-mediated bmf induction for synergistic apoptosis of Eμ-μc/myc-bcl-2 lymphomas in vitro was maintained in the in vivo setting. C57BL/6 mice bearing Eμ-μc/myc-bcl-2, Eμ-μc/myc-bcl-2/bmfshRNA1, Eμ-μc/myc/bmf−/−/bcl-2, and Eμ-μc/myc-bcl-2/IF16shRNA tumors were treated with vehicle, vorinostat, ABT-737, or a combination of vorinostat and ABT-737. In mice bearing Eμ-μc/myc-bcl-2 or Eμ-μc/myc-bcl-2/IF16shRNA lymphomas, treatment with vorinostat or ABT-737 alone caused a slight decrease in WBC numbers and spleen weight (Fig. 3A). The single agent effect on WBC numbers and spleen weights was significantly enhanced following combination treatment with vorinostat and ABT-737 (Fig. 3A). Enhanced induction of apoptosis in mice bearing Eμ-μc/myc-bcl-2 lymphomas treated with the vorinostat/ABT-737 combination compared to single agent treatment was confirmed by TUNEL staining on lymph node tissue (Fig. 3B). The synergistic effect of vorinostat/ABT-737 was almost completely abrogated in mice bearing Eμ-μc/myc-bcl-2/bmfshRNA1 and Eμ-μc/myc/bmf−/−/bcl-2 lymphomas (Fig. 3A).

**Vorinostat increases Bmf expression and induces synergistic apoptosis in tumor cells that constitutively express high levels of Bcl-2**

Having showed that in tumor cells engineered to over-express Bcl-2, treatment with vorinostat increased the levels of Bmf and this was important to "prime" cells for subsequent killing by ABT-737, we wanted to confirm that vorinostat could act in the same manner in a tumor where endogenous Bcl-2 was highly expressed. We previously determined that the mouse breast carcinoma cell line 4T1.2 was relatively resistant to apoptosis induced by single agent vorinostat treatment in vitro and in vivo (27). Western blot analysis revealed that 4T1.2 cells expressed levels of Bcl-2 comparable to the expression level seen in Eμ-μc/myc-bcl-2 lymphomas (Fig. 4A). Treatment of 4T1.2 cells with vorinostat or ABT-737 alone for 24 hours induced minimal apoptosis however synergistic tumor cell death was apparent following combination treatment with ABT-737 and vorinostat (Fig. 4B). Moreover, a time course assay showed that pretreatment of 4T1.2 cells for 16 hours with vorinostat sensitized the cells to ABT-737–induced apoptosis with similar kinetics and magnitude of cell death to those observed in Eμ-μc/myc-bcl-2 lymphomas (Fig. 4C). Bmf mRNA and protein was strongly induced following treatment of 4T1.2 cells with vorinostat with kinetics of gene induction that mirrored those observed in Eμ-μc cells (Fig. 4D and E). Finally, we showed that the combination of vorinostat and ABT-737 was superior in vivo for the treatment of 4T1.2 tumors compared to single agent treatment (Fig. 4F). These data are consistent with the notion that high levels of endogenous Bcl-2 may confer a level of resistance to vorinostat-mediated apoptosis, which can be overcome with prior incubation of cells in vorinostat, followed by the addition of a BH3-mimetic. Effects of tumor microenvironment and/or differences in local drug concentrations in subcutaneous versus lymphoid may account for the combination effect of vorinostat and ABT-737 being less robust in the 4T1.2 system compared to that seen against Eμ-μc/myc-bcl-2 lymphomas.

**Bmf interacts with Bcl-2 and binds Mcl-1 upon addition of ABT-737**

Bmf was originally identified in a yeast 2-hybrid screen using Mcl-1 as bait and co-immunoprecipitated with over-expressed Bcl-2, Bcl-xL, Mcl-1, and Bcl-w in HEK-293 cells (28). BIOCORE analysis using recombinant proteins and Bmf-BH3 peptides indicated that the binding affinity of Bmf was higher for Bcl-2 and Bcl-xL than for Mcl-1 (29). Consistent with these data, we showed that Bmf co-immunoprecipitated with Bcl-2 whereas little or no Bmf was detected following immunoprecipitation of Mcl-1 (Supplementary Fig. S6). Interestingly, treatment of cells with ABT-737 resulted in a decrease in the amount of Bmf that co-immunoprecipitated with Bcl-2 and detectable Bmf that co-immunoprecipitated with Mcl-1 (Supplementary Fig. S6). This shows that Bmf preferentially binds Bcl-2 over Mcl-1. However, ABT-737, which can functionally interact with Bcl-2 but not Mcl-1 (14, 15, 22, 23) appears to effectively compete with Bmf for binding to Bcl-2, resulting in release of Bmf that is subsequently free to interact with Mcl-1.

**Role of the p53 pathway in mediating potent tumor cell apoptosis by vorinostat and ABT-737**

During the course of our experiments we identified an Eμ-μc/myc-bcl-2 lymphoma (designated *Eμ-μc/myc-bcl-2) that displayed considerable resistance to apoptosis induced by the vorinostat/ABT-737 combination (Fig. 5A). Bmf was up-regulated in vorinostat-treated *Eμ-μc/myc-bcl-2 cells indicating that a lack of bmf induction was not responsible for the observed resistance to vorinostat/ABT-737 (Fig. 5B). The parental *Eμ-μc lymphoma retained sensitivity to vorinostat-induced apoptosis (Supplementary Fig. S7A), however, these cells were relatively resistant to apoptosis mediated by etoposide (Supplementary Fig. S7B). Eμ-μc lymphomas have a propensity to inactivate the p53 pathway through mdm2, arf, and p53 loci resulting in chemoresistance in these tumors (30, 31). In comparison to etoposide-sensitive Eμ-μc cells, *Eμ-μc cells expressed significant levels of a higher molecular weight form of p53 that was not induced by etoposide (Supplementary Fig. S7C). Moreover, the *Eμ-μc lymphoma expressed constitutively high levels of p19arf, a phenotype consistent with deletion or functional mutation of p53 (31, 32). We subsequently sequenced p53 from *Eμ-μc cells and identified an
insertion of 33 nucleotides resulting in an 11 amino acid insertion within the C-terminal, oligomerization domain of the protein.

To definitively show that wild-type p53 was required for maximal apoptosis mediated by the ABT-737/vorinostat combination we developed Eμ-myc/p53−/−/bcl-2 and
Figure 4. Vorinostat and ABT-737 induce synergistic death of 4T1.2 cells that constitutively express high levels of Bcl-2. A, the level of Bcl-2 expression in 4T1.2 breast cancer cells compared to Eμ-myc/bcl-2 lymphoma cells was determined by Western blot. Expression of β-actin was used as a loading control. B, 4T1.2 breast cells were incubated in media supplemented with DMSO, 1 μmol/L ABT-737 or 10 μmol/L vorinostat for 24 hours, or were pretreated with 10 μmol/L vorinostat for 16 hours and further incubated with 1 μmol/L ABT-737 for 8 hours. Cell death was assessed by FACS and displayed as percentage annexinV and PI double-positive cells (**, *P < 0.005). C, a synergistic cell death assay was performed with 4T1.2 cells with 10 μmol/L vorinostat and 1 μmol/L ABT-737. Cell death was assessed by FACS and displayed as annexinV and PI double-positive cells. D, qRT-PCR was performed on cDNA samples prepared from 4T1.2 cells incubated under the conditions described in C. Results shown are expressed as fold increase relative to a non-HDACi-regulated control gene RPL32. All data shown are the mean of 3 independent experiments ± SEM. A statistically significant increase in Bmf was observed at the 16-hour timepoint (*P = 0.012). E, western blotting was performed on samples incubated under the conditions described in C and were probed multiple times with antibodies targeting acetylated H4, 4 BH3-only family members (Bmf, Hrk, Bim, and Bad), the multidomain Bax, and actin was used as a loading control. F, BALB/c mice with established subcutaneous 4T1.2 tumors (>6 mm²) were treated with vehicle alone, 200 mg/kg vorinostat, 150 mg/kg ABT-737, or a combination of vorinostat and ABT-737. Ten mice per group were monitored from day 1 of therapy for tumor growth over 15 days (**, *P < 0.05). The data shown is the mean ± SEM.

Eμ-myc/bcl-2/p53shRNA lymphomas (Supplementary Fig. 7C). Apoptosis mediated by the combination of vorinostat and ABT-737 was significantly compromised in Eμ-myc/p53−/−/bcl-2 and Eμ-myc/bcl-2/p53shRNA lymphomas compared to control Eμ-myc/bcl-2 and Eμ-myc/bcl-2/IF16shRNA cells (Fig. 5C). However, robust induction of bmf by vorinostat was retained in Eμ-myc/p53−/−/bcl-2 (Fig. 5D) and Eμ-myc/bcl-2/p53shRNA cells (Fig. 5E) confirming that an intact p53 pathway was not necessary for vorinostat to induce expression of this functionally important BH3-only gene.

To determine if the combination of loss of bmf and p53 had a more pronounced effect on apoptosis induced by the vorinostat/ABT-737 combination than loss of either gene alone, we identified a rare Eμ-myc/bmf−/− lymphoma (termed *Eμ-myc/bmf−/−) with a defect in the p53 pathway as evidenced by loss of detectable p53 protein (in the presence or absence of exposure to etoposide) and increased expression of p19ARF protein (Fig. 6B and C). These cells were relatively resistant to etoposide-induced apoptosis (Fig. 6A) consistent with these cells being defective in p53 function. We subsequently expressed Bcl-2 in *Eμ-myc/bmf−/− cells and determined the effect of vorinostat combined with ABT-737. As expected, Eμ-myc/bcl-2 lymphomas were highly sensitive to the combination of vorinostat and ABT-737 while *Eμ-myc/ bmf−/−/bcl-2 lymphomas with knockout of bmf and loss of
function of p53 were highly resistant to combination treatment (Fig. 6D).

We confirmed that optimum apoptosis induced by vorinostat/ABT-737 required both bmf and p53 using sensitive clonogenic assays. As expected, treatment of Eµ-myc/bcl-2 or Eµ-myc/bcl-2/IFI16shRNA lymphomas with vorinostat and ABT-737 resulted in almost complete loss of clonogenic potential (Fig. 6E). In contrast, the clonogenic potential was partially rescued in Eµ-myc lymphomas with knockdown of bmf (Eµ-myc/bcl-2/bcl-2shRNA) or mutation of p53 (*Eµ-myc/bcl-2) and this rescue was further enhanced in *Eµ-myc/bmf−/−/bcl-2 lymphomas with knockout of bmf and functional inactivation of p53 (Fig. 6E). These data indicate that both bmf and p53 play important functional roles in mediating robust tumor cell apoptosis following combination treatment with vorinostat and ABT-737.

The BH3-only genes puma and noxa are direct transcriptional targets of p53 (33–35) and both genes are elevated in premalignant B cells from Eµ-myc mice (20). As shown in Figure 7A, the expression of noxa mRNA was significantly
showed that the E
phomas with vorinostat and ABT-737 in a time-course assay p53 expression in E
treatment of E
etoposide and Western blotting was performed using specific antibodies against p53 and p19ARF. Expression of a synergistic cell death assay was performed with E
expression was suppressed in *E
idal level of resistance to the combination treatment compared to that observed in *E
m
inhibits the apoptotic and therapeutic activities of diverse HDACi, including vorinostat, panobinostat, romidepsin, and valproic acid (3, 15, 37, 38). Moreover, we showed that the overexpression of prosurvival Bcl-2 family proteins HDACi are a promising class of new anticancer drugs that show single agent clinical activity against a range of hematological malignancies, most notably cutaneous T-cell lymphoma (36). Using syngeneic mouse models of cancer we have shown that overexpression of prosurvival Bcl-2 family proteins inhibits the apoptotic and therapeutic activities of diverse HDACi, including vorinostat, panobinostat, romidepsin, and valproic acid (3, 15, 37, 38). Moreover, we showed that the resistance to vorinostat-induced apoptosis mediated by Bcl-2 or Bcl-xL could be reversed using ABT-737, a small molecule
diminished in p53-defective *E
lymphomas whereas expression of puma was decreased to a lesser extent. In contrast, expression of bmf and bad was not affected in *E
lymphomas in response to incubation with 2.5 μmol/L vorinostat for 16 hours was performed. A specific antibody against Bmf was used and expression of β-actin served as a loading control. C, the functional status of the p53 pathways in *E
expression was assessed by annexinV/PI positive staining and is the mean of 3 independent experiments ±SEM. E, long-term assays were performed on various *E
lymphoma cells pretreated with 2 μmol/L vorinostat for 16 hours followed by further incubation with 0.1 μmol/L ABT-737 for 8 hours. Samples were taken and incubated in soft agar for 7 days. Colony counts are expressed as a percentage of untreated samples. The data shown are the mean ±SEM of at least 4 independent experiments.

Discussion

HDACi are a promising class of new anticancer drugs that show single agent clinical activity against a range of hematological malignancies, most notably cutaneous T-cell lymphoma (36). Using syngeneic mouse models of cancer we have shown that overexpression of prosurvival Bcl-2 family proteins inhibits the apoptotic and therapeutic activities of diverse HDACi, including vorinostat, panobinostat, romidepsin, and valproic acid (3, 15, 37, 38). Moreover, we showed that the resistance to vorinostat-induced apoptosis mediated by Bcl-2 or Bcl-xL could be reversed using ABT-737, a small molecule...
prosurvival protein Mcl-1, which is very poorly inhibited by ABT-737, or due to other events such as phosphorylation of Bcl-2 (22, 23). Moreover, it has been recognized that the mere presence of high levels of Bcl-2 or Bcl-XL does not necessarily mean that such cells are dependent or "addicted" to the activity of these proteins. Indeed, the concept of "apoptotic priming" has been used to explain why certain cells may be either inherently sensitive or resistant to ABT-737 treatment (40). As activation of the intrinsic apoptotic pathway is regulated by the functional interaction between prosurvival Bcl-2 and proapoptotic BH3-only proteins, cells in which prosurvival Bcl-2 family proteins are largely bound by BH3-only proteins are considered to be "primed" for apoptosis mediated by ABT-737 (40). Cells that express prosurvival Bcl-2 proteins at levels that exceed the capacity of endogenous BH3-only proteins to bind them may be considered to be "unprimed" and therefore less sensitive to single agent ABT-737 treatment (40). It therefore follows that an agent capable of increasing the expression or activity of BH3-only proteins, and/or decreasing the expression/activity of prosurvival Bcl-2 family members, may sensitize "unprimed" tumor cells to ABT-737-mediated apoptosis (Supplementary Fig. S8A). Consistent with this model, combining ABT-737 with proapoptotic stimuli such as conventional chemotherapeutic drugs, γ-irradiation and novel "targeted" small molecule and biological agents can induce synergistic apoptosis in tumor cells that are relatively resistant to single agent treatment (39).

HDACi and ABT-737 used in combination can induce synergistic tumor cell apoptosis (15, 41). We have showed that vorinostat-mediated induction of bim may be important for the single agent apoptotic and therapeutic activity of vorinostat (3) and bim was important for the combined activities of suberoyl bis-hydroxamic acid (SBHA) and ABT-737 in human tumor cell lines (41). However, as we clearly showed herein, knockout of bim had no effect on the ability of the vorinostat/ABT-737 combination to kill Eμ-myc/bcl-2 lymphomas even though bim was induced by vorinostat at both the mRNA and protein level. In contrast, knockout or knockdown of bmf impaired synergistic tumor cell apoptosis induced by vorinostat and ABT-737. In Eμ-myc/bcl-2 lymphomas, Bmf was robustly sequestered by Bcl-2 however, upon addition of ABT-737, Bmf was rapidly released from Bcl-2, presumably as a result of avid binding of ABT-737 to Bcl-2. Bmf was then free to interact with Mcl-1 and possibly other BH3-only proteins, and/or decreasing the expression/activity of endogenous BH3-only proteins to bind them may be considered to be "unprimed" and therefore less sensitive to ABT-737 treatment (40). An agent capable of increasing the expression or activity of BH3-only proteins, and/or decreasing the expression/activity of prosurvival Bcl-2 family members, may sensitize "unprimed" tumor cells to ABT-737-mediated apoptosis (Supplementary Fig. S8A). Consistent with this model, combining ABT-737 with proapoptotic stimuli such as conventional chemotherapeutic drugs, γ-irradiation and novel "targeted" small molecule and biological agents can induce synergistic apoptosis in tumor cells that are relatively resistant to single agent treatment (39).

Mechanism of Tumor Cell Death by HDACi and ABT-737

ABT-737 often has weak single agent activity against a range of tumor cell lines even though these cells frequently express high levels of Bcl-2/Bcl-xL (39). Such cells may be insensitive to ABT-737 due to concomitantly high levels of the prosurvival protein Mcl-1, which is very poorly inhibited

**Figure 7.** Knockout of noxa inhibits robust apoptosis induced by the combination of vorinostat and ABT-737. A, qRT-PCR analysis was performed on cDNA prepared from untreated Eμ-myct/bcl-2 and *Eμ-myct/bcl-2 cells using primers against the p53-regulated genes bad, noxa, and puma. The bmf primer set served as a control. Results shown are expressed as fold increase relative to a non-HDACi-regulated control gene RPL32 and is the mean of 3 independent experiments ±SEM. B, Eμ-myct/bcl-2, *Eμ-myct/bcl-2, and Eμ-myct/noxa−/−/bcl-2 lymphoma cells were incubated ±20 nmol/L etoposide for 24 hours and assessed for p53 pathway status by Western blotting using specific antibodies towards p53/C6-actin served as a loading control. C, a synergistic cell death assay with Eμ-myct/Bcl-2, *Eμ-myct/Bcl-2, and Eμ-myct/noxa−/−/bcl-2 lymphoma cells was analyzed for sensitivity to vorinostat and ABT-737 in combination. Cell death was assessed by FACS analysis of positive annexin/V/PI staining (**, P < 0.05).

inhibitor of Bcl-2 proteins (15). Herein, we have used mouse genetics to decipher the apoptotic proteins and pathways necessary for vorinostat and ABT-737 to kill tumor cells overexpressing Bcl-2.

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by ABT-737, or due to other events such as phosphorylation of Bcl-2 (22, 23). Moreover, it has been recognized that the mere presence of high levels of Bcl-2 or Bcl-xL does not necessarily mean that such cells are dependent or "addicted" to the activity of these proteins. Indeed, the concept of "apoptotic priming" has been used to explain why certain cells may be either inherently sensitive or resistant to ABT-737 treatment (40). As activation of the intrinsic apoptotic pathway is regulated by the functional interaction between prosurvival Bcl-2 and proapoptotic BH3-only proteins, cells in which prosurvival Bcl-2 family proteins are largely bound by BH3-only proteins are considered to be "primed" for apoptosis mediated by ABT-737 (40). Cells that express prosurvival Bcl-2 proteins at levels that exceed the capacity of endogenous BH3-only proteins to bind them may be considered to be "unprimed" and therefore less sensitive to single agent ABT-737 treatment (40). It therefore follows that an agent capable of increasing the expression or activity of BH3-only proteins, and/or decreasing the expression/activity of prosurvival Bcl-2 family members, may sensitize "unprimed" tumor cells to ABT-737-mediated apoptosis (Supplementary Fig. S8A). Consistent with this model, combining ABT-737 with proapoptotic stimuli such as conventional chemotherapeutic drugs, γ-irradiation and novel "targeted" small molecule and biological agents can induce synergistic apoptosis in tumor cells that are relatively resistant to single agent treatment (39).

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Expression of wild-type p53 was important for tumor cell death mediated by the vorinostat/ABT-737 combination. Loss of function of p53 did not affect vorinostat-induced expression of BH3-only genes, however, the basal level expression of p53-target genes puma, and in particular noxa, was significantly reduced. Noxa has exquisite binding specificity for Mcl-1 whereas Puma has been showed to more broadly bind
numerous prosurvival Bcl-2 proteins and additionally interact with, and directly activate, Bax and Bak (40). Accordingly, we posit that decreased expression of puma and noxa through loss of p53 function would likely raise the "apoptotic threshold" that is mediated primarily through the intrinsic pathway (Supplementary Fig. S8B). Clearly p53 has tumor suppressor and apoptotic functions that are independent of puma and/or noxa (42), however, in the context of synergistic apoptosis mediated by vorinostat and ABT-737, loss of noxa alone was sufficient to blunt the apoptotic effects of the combination. The ability of Noxa to selectively interact with Mcl-1 with high affinity (29) likely underpins the important role of noxa in mediating optimal tumor cell death by vorinostat and ABT-737. Indeed, it appears that noxa plays an important role in synergistic apoptosis mediated by ABT-737 and a range of other anticancer agents. For example, NOXA but not BIM was important for synergistic death of human melanoma cells mediated by ABT-737 combined with antimelanoma drug imiquimod even though both BH3-only genes were induced by imiquimod (43).

The requirement for both bmf and p53 to mediate the most potent apoptotic response to the vorinostat/ABT-737 combination was shown using Eμ-myc/Bcl-2 lymphomas with knockout or knockdown of bmf and a concomitant loss of p53 function. Our data are consistent with a model whereby Bmf induction is important for vorinostat to "prime" cells for apoptosis mediated by ABT-737, whereas p53 activity lowers the "apoptotic threshold" by maintaining elevated levels of noxa and possibly puma. These data provide important mechanistic insight into the molecular events necessary for vorinostat to function synergistically with ABT-737. Moreover, our finding that Bmf was more important than was Bim, for priming of Eμ-myc/Bcl-2 lymphoma cells by vorinostat for ABT-737–induced apoptosis was unexpected, given that Bim has broader binding specificity for prosurvival Bcl-2 proteins than does Bmf and, unlike Bmf, Bim has been reported to directly activate Bax and Bak (40). This indicates that it is not simply induction of any BH3-only gene that "primes" cells for apoptosis mediated by ABT-737, but rather specific BH3-only proteins, or combinations of BH3-only proteins may be required for this process. This hypothesis is in agreement with recent studies by Letai and colleagues that described "priming" as specific competitive interactions between 2 subclasses of BH3-only proteins based on their ability to directly bind Bak and Bax (activators) or compete for binding of prosurvival Bcl-2 family members (sensitizers). Letai and colleagues suggested that primed cells undergo apoptosis via specific release of activator BH3-only members allowing oligomerization of Bax and/or Bak (40).

Different ABT-737–sensitizing agents likely engage specific BH3-only proteins to synergize with ABT-737, presumably in a cell-type specific manner. Thus, in different cell types across and within species there are particular requirements for expression of activator BH3-only members such as Bim or sensitizer BH3-only members such as Bmf, with the relative number and levels of expression of BH3-only members from each subclass determining the state of cellular priming. This was apparent when comparing our results with those of Chen and colleagues who showed a requirement for Bim in mediating synergistic apoptosis by ABT-737 and SBHA (41), whereas we showed that Bim was dispensable for apoptosis induced by the vorinostat/ABT-737 combination. In addition, knockdown of NOXA attenuated the combined apoptotic effects of CPT-11 and ABT-737 in HCT16 cells but not in HT-29 cells whereas knockdown of NOXA equivalently suppressed the synergistic effects of bortezomib and ABT-737 in both cell lines (44).

These preclinical studies provide the most comprehensive functional link between tumor genotype and the apoptotic and therapeutic effects of HDACi combined with ABT-737. Our work provides the basis for the rational use of this combination in the clinic and identifies potential biomarkers such as HDACi-mediated induction of bmf and p53 status to putatively predict likely therapeutic efficacy.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone: commercial research grant, Merck and Co. The other authors declared no potential conflicts of interest.

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Deciphering the Molecular Events Necessary for Synergistic Tumor Cell Apoptosis Mediated by the Histone Deacetylase Inhibitor Vorinostat and the BH3 Mimetic ABT-737

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