The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim

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Abstract

Previously, we showed that the proteasome inhibitor bortezomib/Velcade (formerly PS-341) synergizes with the protein tumor necrosis factor α–related apoptosis-inducing ligand (TRAIL), a ligand for certain death receptors, to induce apoptosis in cell lines derived from prostate and colon cancers. Because apoptosis is often triggered by BH3-only proteins of the Bcl-2 family, we have explored the hypothesis that bortezomib contributes to the apoptosis by up-regulating their levels. Indeed, bortezomib induced increases of Bik and/or Bim in multiple cell lines but not notably of two other BH3-only proteins (Puma and Bid) nor other family members (Bax, Bak, Bcl-2, and Bcl-x_L). The increase in Bik levels seems to reflect inhibition by bortezomib of its proteasome-mediated degradation. Importantly, both Bik and Bim seem central to the proapoptotic function of bortezomib because mouse embryo fibroblasts in which the genes for both Bik and Bim had been disrupted were refractory to its cytotoxic action. Similarly, the synergy between bortezomib and TRAIL in killing human prostate cancer cells was impaired in cells in which both Bik and Bim were down-regulated by RNA interference. Further evidence that bortezomib acts through the mitochondrial pathway regulated by the Bcl-2 family is that deficiency for APAF-1, which acts downstream of Bcl-2, also blocked its apoptotic effect. These results implicate BH3-only proteins, in particular both Bik and Bim, as important mediators of the antitumor action of bortezomib and establish their role in its enhancement of TRAIL-induced apoptosis. [Mol Cancer Ther 2005;4(3):443–9]

Introduction

The proteasome inhibitor bortezomib/Velcade (formerly PS-341) has recently entered clinical practice as a treatment for multiple myeloma and is undergoing clinical trials for other types of cancer (1). Its mode of action is not established but is very likely to involve promotion of apoptosis (2, 3). Diverse mechanisms have been proposed. Some results suggest that bortezomib might act through the ''death receptor'' pathway, in which extracellular ligands promote apoptosis through the activation of caspase-8 (4). Gene expression studies in cultured cells have shown that bortezomib treatment increases the level of the mRNAs for a number of proapoptotic proteins, including that of the death receptor DR5, which can be engaged by the tumor necrosis factor α–related apoptosis-inducing ligand (TRAIL; refs. 5, 6). Moreover, bortezomib has been reported to reduce levels of c-FLIP (7), which counters the activation of caspase-8, or to increase the activation of caspase-8 and its target Bid through mechanisms not involving c-FLIP (6). On the other hand, other results implicate the intrinsic pathway to apoptosis, in which the Bcl-2 protein family and mitochondria play key roles (8). Thus, bortezomib has been reported (a) to stabilize and activate the tumor suppressor p53 (9), which acts upstream of Bcl-2; (b) to stabilize IκB and thereby decrease the antiapoptotic effects of nuclear factor κB (10), which are frequently mediated through the Bcl2 family; and (c) to damage mitochondria through generation of reactive oxygen species, a response attenuated by Bcl-2 (11).

Such findings favor the view that bortezomib and perhaps other modes of proteasome inhibition promote apoptosis at least in part through the pathway regulated by the Bcl-2 family (2, 3). As well as members that promote cell survival (e.g., Bcl-2 and Bcl-x_L), this family includes two proapoptotic groups: the eight or more proteins termed ''BH3-only'' because they bear only the small BH3 protein-interaction domain (e.g., Bik, Bim, Bid, and Puma) serve as triggers for the apoptotic signal, whereas Bax and Bak act downstream to impose apoptosis, probably mainly through permeabilization of mitochondria (8, 12, 13). We showed previously (6) that bortezomib synergizes with TRAIL to induce apoptosis in prostate and colon cancer cell lines. Pertinently, mitochondrial permeabilization was

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implicated by the early release of cytochrome *c* and second mitochondrial activator of caspases, proteins that promote the activation of caspase-9 via the scaffold protein Apaf-1, or antagonize the inhibitory effect of XIAP on caspase-9 respectively. Furthermore, in mouse embryonic fibroblasts (MEF), Bax and Bak were required for the apoptosis induced by bortezomib alone, and the absence of Bak protected cells against death induced by bortezomib combined with TRAIL (6).

As TRAIL has promise as an anticancer agent (4), we have explored further how bortezomib sensitizes cells to its action. Because the level of BH3-only proteins often seems a critical determinant of whether apoptosis ensues (8, 12, 14), our previous findings (6) have stimulated us to test whether bortezomib might act by increasing the level of certain BH3-only proteins. In keeping with that hypothesis, we report here that bortezomib induces increased levels of the BH3-only proteins Bik and Bim in a number of cancer cell lines and in MEFs. Bim is known to be required for apoptosis induced in hematopoietic cells by several types of cytotoxic stimuli and to participate in the developmentally programmed death of several cell types (12, 14, 15). Less is known about Bik, but the mouse gene (previously denoted Blk) is expressed in diverse cell types (16), including some cancer cell lines (17), and Bik mutations have been reported in some human B cell lymphomas (18). Significantly, we show that suppression of expression of both these proteins in MEFs or prostate cancer cells inhibits the apoptosis induced by bortezomib or by combined bortezomib/TRAIL treatment.

Materials and Methods

Cell Lines and Reagents

MEFs, Du145, PC-3, and Alva human prostatic cancer cell lines, 293T, and MCF-7 human breast cancer cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA), 100 units/mL penicillin, and 100 μg/mL streptomycin. The human prostatic cancer cell line LNCaP was grown in RPMI 1640 (Invitrogen) with glutamine and the same supplements. Cultures were maintained at 37°C at 95% humidity. Antibodies were obtained from the following sources: Bik from Santa Cruz Biotechnology (Santa Cruz, CA); Bcl-2, Bax, and Bak from Upstate Biotechnology (Lake Placid, NY); Bim from Stressgen Bioreagents (Victoria, British Columbia, Canada); poly(ADP-ribose) polymerase from Trevigen (Gaithersburg, MD); glyceraldehyde 3′-phosphate dehydrogenase from Chemicon (Temecula, CA); and HA, FLAG, and FLAG agarose from Sigma-Aldrich Co. (St. Louis, MO). Recombinant human TRAIL was prepared as described previously (6). Bortezomib (Velcade, formerly PS-341) was a gift of Millenium, Inc. (Cambridge, MA).

Cell Viability Assays

Assays employing 4′,6-diamido-2-phenylindole (DAPI) were done as described previously (6). Assays measuring cell survival were done as follows: cells were plated in 6- or 24-well plates. Following an 18-hour treatment with bortezomib and/or TRAIL, nonviable cells were aspirated from the plates and the remaining attached cells rinsed twice with PBS. Depending on the cell density, plates were treated in either of two ways: (a) the cells were trypsinized, and cell number determined by hemacytometer; or (b) the cells were fixed on the plates with 2% formaldehyde/0.2% glutaraldehyde in PBS for 5 minutes, followed by two rinses with PBS. The cells in a series of adjacent fields from the same region of each well were then counted microscopically. Where possible, both methods were used and equivalent results were obtained.

Plasmids and Transfection

Human Bik was cloned from cDNA that had been reverse transcribed from LNCaP cell total RNA, using as 5′ primer 5′-CCATGGATCCACCATGGACTACAAGGACGACGATGACAAGATGTCTGAAGTAAGACCCCTCTCC, which contains the sequence coding for the FLAG epitope tag and 3′ primer 5′-AGTAGTGCGGCCGCTCACTTGAGCAGGTGCAGG. Integrity of the constructs was verified by DNA sequencing (University of Colorado Cancer Center DNA Sequencing and Analysis Core). The constructs expressing RNA interference (RNAi) directed against Bik from the human U6 promoter (target sequence 5′-CATAATGAGGTTCTGGAGA), and control RNAi, were made using the Silencer Express kit (Ambion, Austin, TX) and inserted into the pcDNA3 vector (Stratagene, La Jolla, CA), following excision of sequences for the cytomegalovirus promoter. The construct expressing RNAi against Bim was generated in the pSuper vector as described (19). It contains the sequence 5′-TGATGTAAGTTCTGAGTGGTG, which is common to all known Bim mRNA isoforms and is 100% conserved in human Bim. A hygromycin resistance cassette was added to this construct for generating stable cell lines. Transient transfections were done with Effectene (Qiagen, Valencia, CA) following the manufacturer's instructions. Stable cell lines were derived by G418 (anti-Bik, LNCaP) or hygromycin (anti-Bim, MCF-7) selection.

Reverse Transcription-PCR

cDNA was synthesized from total RNA (20) with SuperScript II and random primers (Invitrogen) following the manufacturer's instructions. Primers for Bik were 5′-GGAGACCCTCCTGTATGAGC (forward) and 5′-ACCTGTTCGCAGGACACC (reverse) and were designed with Primer3 software (21).

Immunoprecipitation

Immunoprecipitation of Bik was done as follows: cells were lysed in >5 volumes of lysis buffer [50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 20 mmol/L EDTA, 20 mmol/L NaF, 0.5% Igepal (NP40), 40 mmol/L β-glycerophosphate, 0.2 mmol/L phenylmethylsulfonylfluoride, and protease inhibitor cocktail (Sigma)]. Lysates were rotated overnight at 4° with either agarose beads to which anti-FLAG epitope antibody had been covalently coupled (Sigma), or protein-A-protein G agarose beads (Calbiochem/EMD Biosciences, San Diego, CA) to which

anti-Bik antibody had been bound by incubation. After extensive rinses with lysis buffer, bound proteins were eluted with 100 mmol/L glycine (pH 2.3), neutralized, and processed for Western blotting.

Cell Sorting and Western Blotting

Cells were sorted on the basis of green fluorescent protein (GFP) expression at the University of Colorado Cancer Center Flow Cytometry Core. Western blotting was done as described (6).

Mouse Embryo Fibroblasts

Fibroblasts were prepared from mouse embryos in which the genes for Bim, Blk, or Apaf-1 had been disrupted by homologous recombination (9, 16). Fibroblasts doubly deficient in Bim and Blk were obtained from embryos obtained from crosses between homozygous *bim* and *bik*-deficient mice.

Statistics

Data were analyzed first by ANOVA. Pairwise comparisons were then done with Bonferroni *t* test (22).

Results

Regulation of the Level of BH3-Only Proteins by Bortezomib

We showed previously that bortezomib treatment of LNCaP cells did not affect the levels of either Bax or Bak (3). To examine the possibility that certain proteins of the BH3-only subfamily might be up-regulated by bortezomib, we probed Western blots from a panel of seven cell lines including prostate, colon, and breast cancer cell lines with antibodies directed against diverse proteins of the Bcl-2 family (Fig. 1A). Except for a slight rise in Bid in two lines (HC-4 and MCF-7), bortezomib treatment did not affect the levels of Bcl-xL, Bcl-2, Bax, Bak, or PUMA. In striking contrast, the level of the BH3-only protein Bik was elevated by the drug treatment in every cell line examined. Moreover, the level of Bim rose in HC-4 colon cancer cells (Fig. 1A), MEFs (Fig. 1C), and MCF-7 human breast cancer cells (Fig. 1A). The fold changes varied among cell lines but was as high as 4.2-fold in Du145 cells. The kinetics of induction of Bik in LNCaP cells (Fig. 1B) revealed that significant elevation was evident after only 1 hour of exposure to bortezomib, and the level continued to increase for several hours, indicating that Bik might well have a role in the induction of apoptosis. Rapid elevation was also observed in HC-4 and PC-3 cells (data not shown). As measured by the number of cells occupying the sub-G_1 peak on fluorescence-activated cell sorting, 17 hours of bortezomib treatment induced relatively little cell death in these lines (LnCaP, 8.8% increase in cell death when compared with control untreated cells [all data is the average of triplicate determinations]; MCF-7, 0%; 293T, 1%; PC-3, 6.71%; HC-4, 3.16%; Du145, 1%). Longer incubations killed significantly more cells in all lines.

To determine whether Bik up-regulation instead reflected inhibition of proteasome-mediated protein degradation (3), we tested whether ubiquitinated forms of the protein accumulated in response to bortezomib. To do so, a FLAG-Bik expression construct was transfected into 293T cells together with a HA-tagged ubiquitin expression construct. Bik was then immunoprecipitated from the transfected cells, subjected to electrophoresis and shown by Western blotting using antibodies directed against HA and Bik. Figure 2 shows that both antibodies detected higher molecular weight bands specifically in Bik-transfected cells. The increase in their intensity following bortezomib treatment suggests that Bik is normally degraded by the proteasome via the standard ubiquitin-mediated pathway. Very little HA-specific staining was detected at the molecular weight expected for monoubiquitinated Bik ~28 kDa. As suggested by others (23), this result could reflect the preferential utilization of monoubiquitinated proteins in signaling pathways distinct from degradation.

Thus, bortezomib seems to up-regulate Bik by inhibiting its proteasomal degradation, and recent work suggests that the abundance of Bim can also be regulated by this mechanism (24, 25).

Central Roles of Bik and Bim in Mediating Apoptosis by TRAIL and Bortezomib

We showed previously (6) that bortezomib treatment increased sensitivity to TRAIL-induced apoptosis in LNCaP prostate cancer cells, HC-4 Bax-negative colon cancer cells, and MEFs. MEFs containing both Bax and Bak undergo apoptosis when treated with bortezomib alone. As

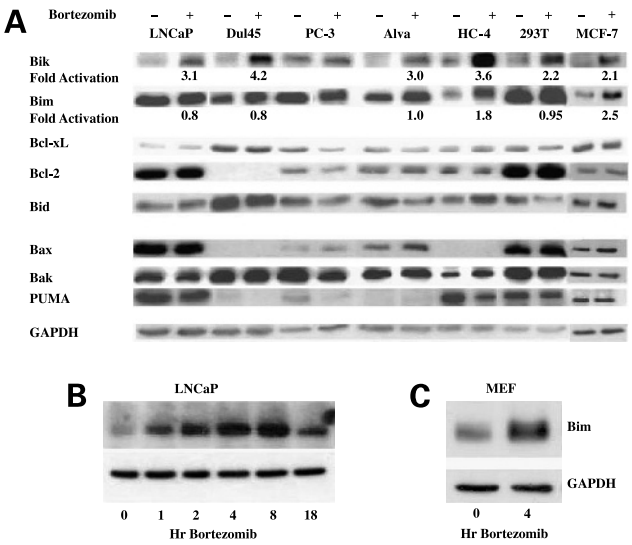

Figure 1. Effects of bortezomib treatment on the levels of members of the Bcl-2 protein family. **A,** the levels of Bcl-2, Bcl-xL, Bax, Bak, PUMA and Bik, Bid, and Bim proteins were assessed in the indicated cell lines. Cells were incubated with 1 μmol/L bortezomib or vehicle for 17 h, lysed, and prepared for Western blot analysis with the indicated antibodies. To quantitate these Western blot bands, regions of interest were defined around the bands. Kodak ID software was used to measure the sum intensity in these regions. The fold-activation reported was determined by dividing the value obtained from the treated cells by those for the control untreated. **B,** LNCaP cells were incubated with 1 μmol/L bortezomib for the indicated times and processed for Western blotting for Bik and GAPDH as a loading control. **C,** MEF were incubated with 1 μmol/L bortezomib for 4 h and processed for Western blotting for Bim and GAPDH as a loading control.

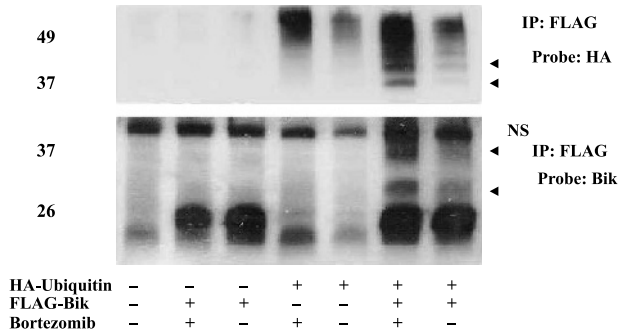

Figure 2. Regulation of Bik protein levels by bortezomib. 293T cells were transfected with expression constructs for HA-ubiquitin and FLAG-Bik in the indicated combinations. The transfected cells were treated with 1 μmol/L bortezomib for 24 h. Lysates of the cells were incubated with anti-FLAG antibody coupled to agarose beads and bead eluates processed for Western blotting as described in Materials and Methods. *Top,* probed with antibody to the HA-tagged ubiquitin. *Arrows,* bands (most prominent in bortezomib-treated cells) specific to cells expressing both the Bik and ubiquitin constructs. *Bottom,* probed with antibody to Bik. Nonspecific band appearing in all preparations (*NS*). *Arrows,* bands specific to cells expressing both the Bik and ubiquitin constructs.

reviewed elsewhere (8, 12), it is well established that overexpression of BH3-only proteins such as Bim and Bik promotes apoptosis (e.g., refs. 26–28). To determine whether the elevated levels of Bik and/or Bim described above contributed to TRAIL sensitivity or to bortezomib-induced apoptosis, we have taken two genetic approaches. To analyze the cancer cell lines, we have generated derivative lines in which Bik or Bim expression has been down-regulated by RNAi via the synthesis of small hairpin RNAs (shRNA; ref. 29). For the MEFs, we have analyzed cells bearing homozygous disruptions of *bim* (15), *bik* (16), or both genes.

Because Bim was not induced by bortezomib in the prostate cancer cell lines examined, we evaluated other malignant cell lines and found significant induction of Bim by bortezomib treatment in MCF-7 cells (Fig. 1A). MCF-7 breast cancer cells were stably transfected with a construct expressing a shRNA directed against Bim. Figure 3A (*top*) shows that the elevation of Bim levels in wild-type cells by bortezomib was almost entirely suppressed by RNAi. The effect seems to be specific, because induction of Hsp-70 by bortezomib was unaffected, whereas Bcl-2 levels remained stable (Fig. 3A), and a point mutation in the hairpin used ablates the suppression by this RNAi.[3] A preliminary experiment using nuclear DAPI uptake as a marker for apoptosis suggested that bortezomib-induced apoptosis in the cell line expressing RNAi did not differ significantly from wild-type MCF-7 after 24 hours (Fig. 3A, *bottom*). To evaluate further the effect of lowering Bim on the ability of bortezomib to enhance TRAIL-mediated apoptosis, we counted the cells remaining attached to the culture dish after combined treatment as an assay for cell survival. The cell line expressing Bim RNAi remained sensitive to TRAIL (Fig. 3B).

[3] P. Bouillet et al., unpublished results.

Similar experiments were conducted using LNCaP prostate cancer cells stably transfected with a shRNA construct directed against Bik. As shown in Fig. 3C, the bortezomib-induced elevation of Bik levels was strongly suppressed in these cells. However, there was no qualitative change in the extent of poly(ADP-ribose) polymerase cleavage resulting from treatment with TRAIL, or the combination of bortezomib and TRAIL, at the concentrations used. Nevertheless, RNAi against Bik can enhance cell survival (see below).

Unlike LNCaP cells, MEFs can be killed by bortezomib alone, as well as by the drug combination. Therefore, the MEFs derived from mice rendered deficient in Bim, Bik, or both proteins by homologous recombination (15, 16) provided an independent approach for evaluating whether either protein was essential for the apoptosis elicited by the drugs. Wild-type and mutant MEFs were treated with bortezomib, TRAIL, or the combination for 18 hours. Cells were then detached from the plates with trypsin and scored for the uptake of DAPI by fluorescence microscopy (Fig. 4). Notably, in the response to bortezomib alone, the MEFs deficient in both Bim and Blk showed <60% of the apoptosis given by the wild-type cells ($P < 0.001$). With that stimulus alone, even the MEFs lacking only Bik showed significantly less death than the wild-type cells ($P < 0.01$), but the absence of Bim alone seemed to have no statistically significant effect. With both bortezomib and TRAIL, apoptosis was high with the cells of all the genotypes, and although the extent seemed slightly lower in the MEFs lacking both Bim and Bik, the decrease was not statistically significant. Overall, the data derived from MEF, MCF-7, and LNCaP cells suggested that, although antiapoptotic effects of suppressing expression of Bim or Bik alone might be difficult to detect, suppression of both together might well convey significant protection. We tested this idea in LNCaP cells by using RNAi to down-regulate expression of both proteins. Control LNCaP cells (expressing an shRNA construct with no effect on Bik levels) and the LNCaP cell line stably expressing a shRNA directed against Bik were transiently transfected with a construct expressing either GFP alone, or GFP plus the anti-Bim shRNA shown to be effective in MCF-7 cells. Twenty-four hours later, GFP-positive cells were isolated by fluorescence-activated cell sorting and plated. After a recovery time of 10 to 12 hours, the cells were treated with bortezomib plus graded concentrations of TRAIL for 12 to 18 hours, then assayed for apoptosis using detachment as a marker. A representative experiment is shown in Fig. 5A. At all TRAIL concentrations tested, the cells expressing shRNAs against both Bim and Bik showed significantly greater survival than those expressing each single shRNA ($P < 0.05$ at 5 ng/mL TRAIL). Moreover, at the highest TRAIL concentration (100 ng/mL), inhibiting expression of either Bim or Bik alone also provided significant protection. However, because most cells were undergoing apoptosis at this concentration of TRAIL, no differences in the extent of poly(ADP-ribose) polymerase cleavage were evident (cf. Fig. 3C).

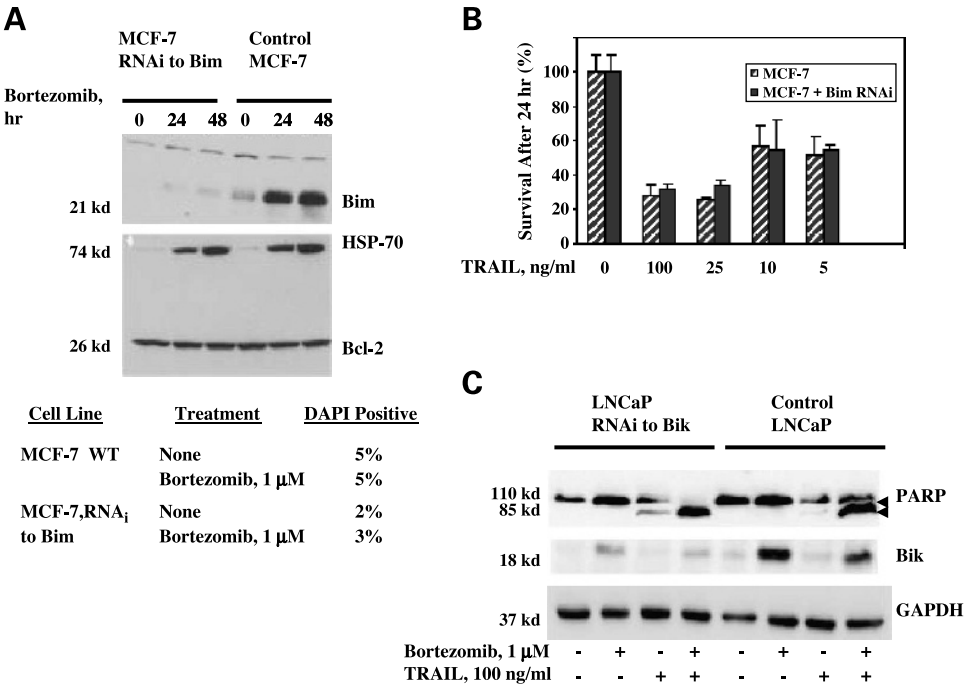

Figure 3. Effectiveness of RNAi directed against Bim and Bik in stable cell lines. **A,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 μmol/L bortezomib for the indicated times. Cell lysates were analyzed by Western blotting for the indicated proteins. *Bottom,* Cells treated with 1 μmol/L bortezomib for 24 h were scored for nuclear DAPI uptake as described in Materials and Methods. **B,** MCF-7 human breast cancer cells stably expressing a shRNA against Bim, and the parental cell line, were treated with 1 μmol/L bortezomib and the indicated TRAIL concentrations for 24 h. Surviving cells were scored as described in Materials and Methods. **C,** LNCaP human prostatic cancer cells stably expressing a shRNA against Bik, and the parental cell line, were treated with bortezomib and TRAIL as indicated for 6 h. Cell lysates were analyzed for the indicated proteins by Western blotting.

Taken together, these results on BH3-only proteins suggest that the mitochondrial pathway of cell death plays a critical role in the ability of bortezomib to sensitize cells to TRAIL-induced cell death. If so, the proapoptotic effect should be suppressed by elimination of a critical downstream effector of mitochondrial disruption, such as the caspase-9 activator Apaf-1. To evaluate this, we studied the response of MEFs deficient in Apaf-1 to these agents (Fig. 5B). Clearly, the absence of Apaf-1 markedly decreased the ability of bortezomib to kill MEFs. At the TRAIL dose used in these studies (1 μg/mL), there was also a small but statistically significant ($P < 0.05$) decrease in the sensitivity of the cells to the combination treatment. This result supports the notion that both the intrinsic and extrinsic pathways are important for TRAIL-mediated apoptosis in MEFs.

Discussion

In this paper, we have shown that bortezomib treatment of a number of cancer cell lines induces increases in the levels of the BH3-only proteins Bik and Bim. Moreover, suppression of this induction, particularly of Bik, results in increased resistance of the cells to apoptosis caused by either bortezomib alone (for MEFs) or, in the case of the LNCaP cells, which are not killed by bortezomib alone, by the combination of TRAIL plus bortezomib. In addition, we found that concomitant suppression of Bim and Bik in both MEFs and LNCaP cells resulted in significantly more resistance than suppression of Bim or Bik individually.

More than eight BH3-only proteins have been described thus far in mammalian cells (8), and gene targeting has been used to clarify the apoptotic roles of six of them: Bim (15), Bad (30), Bid (31), Bik (16), Puma, and Noxa (32). Developmental lesions in such knockout mice range from dramatic (Bim) to as yet undetected (Bik). It seems likely, as is the case for Bax and Bak, that the functions of some BH3-only proteins overlap and hence that specific roles for such proteins appear only when more than one of the genes is suppressed. For example, we have recently found that male mice deficient in both Bim and Bik are sterile,[4] whereas animals deficient in Bim or Bik alone have normal fertility. In accord with redundant function, we observed greatest resistance to apoptosis when expression of both Bim and Bik expression was suppressed.

As yet it is not entirely clear how BH3-only proteins activate the apoptotic machinery or how much their functions overlap (13). There is wide agreement that association of BH3-only proteins with antiapoptotic Bcl-2 family members is a critical step, and that the relative level of these opposing factions is important. In response to bortezomib treatment, we found that levels of Bcl-2 and Bcl-x_L did not change (Fig. 1), although Mcl-1 levels were elevated (data not shown), in accord with its reported regulation by the proteasome (33). It is possible that the binding affinities of Bcl-2 prosurvival family members to various BH3-only proteins varies widely, and hence that the response to an apoptotic stimulus depends upon the precise cellular composition of these two factions (34). In addition to binding prosurvival family members and thus

[4] L. Coultas et al., unpublished results.

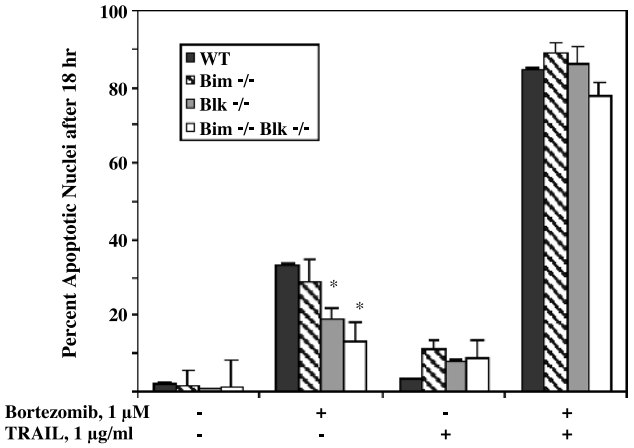

Figure 4. Bortezomib and TRAIL-mediated apoptosis in Bim and/or Bik-deficient MEFs. MEFs were treated as indicated for 18 h and scored for nuclear DAPI uptake as described in Materials and Methods. *Columns,* mean of nuclei and cell counts from two independent experiments; *bars,* SD. *, $P < 0.01$, statistically significant deviation from wild-type value.

releasing their inhibition of Bax and Bak activation, it has been proposed that certain BH3-only proteins, such as Bid (35), can directly interact with Bax and Bak to promote apoptosis.

Preliminary experiments using reverse transcription-PCR and Bik promoter-CAT constructs suggested that bortezomib did not augment Bik transcription in LNCaP or HC-4 cells (data not shown). Our demonstration that Bik is ubiquitinated (Fig. 2) suggests that its rapid accumulation in many cell lines in response to bortezomib is due to stabilization consequent to proteasome inhibition. Others have reported accumulation of Bik in the presence of other proteasome inhibitors (27, 36). Of interest, in one study Bik accumulated to much higher levels in cell lines which overexpressed Bcl-x_L, suggesting that Bcl-x_L served to sequester Bik (27). We observed no correlation, however, between the level of Bcl-2 or Bcl-x_L and the level of Bik induced by bortezomib (cf. Fig. 1A).

Importantly, we showed that MEFs deficient in Bik or both Bim and Bik were significantly resistant to bortezomib-induced apoptosis (Fig. 4). Similarly, LNCaP prostate cancer cells in which both Bim and Bik had been suppressed were resistant to TRAIL-induced apoptosis in the presence of bortezomib (Fig. 5A). We showed previously (6) that enhanced cleavage of caspase-8 and Bid contributed to the synergy between bortezomib and TRAIL in inducing apoptosis. The present results suggest that an additional mechanism is bortezomib-mediated increases of the BH3-only proteins Bik or Bim. When these proteins reach a threshold level, they presumably can neutralize antiapoptotic proteins of the Bcl-2 family and thereby allow activation of Bax and/or Bak, leading to permeabilization of mitochondria and activation of caspase-9 (8). This mechanism is consistent with the marked potentiation of bortezomib on the apoptotic action of drugs such as doxorubicin (37). Thus, bortezomib apparently can promote apoptosis through both this pathway and that involving enhanced Bid cleavage. The relative importance of the intrinsic and extrinsic pathways in its action may

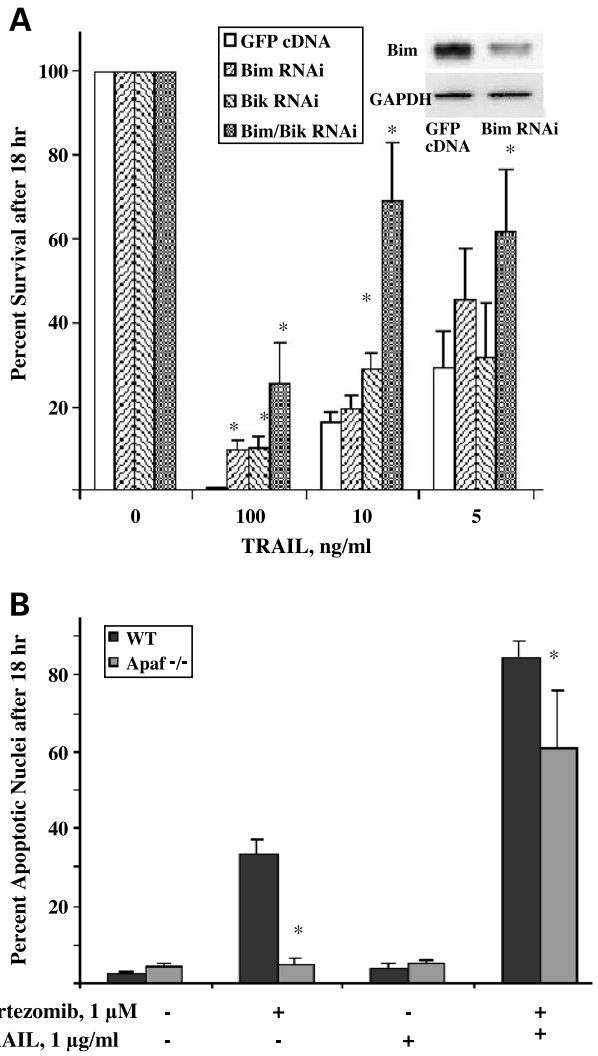

Figure 5. A, Protection against TRAIL-mediated apoptosis by RNAi directed against Bim and/or Bik. LNCaP human prostatic cancer cells stably expressing a shRNA directed against Bik were transiently transfected with constructs expressing GFP and a shRNA directed against Bim, or the cDNA encoding GFP alone. As a control, LNCaP cells stably expressing an RNAi with no effect on Bik or Bim levels were transiently transfected with constructs expressing the cDNA for GFP and or an shRNA directed against Bim, or the cDNA for GFP alone. Cells expressing GFP were sorted by FACS, plated, and treated with TRAIL at the indicated concentrations in the presence of 1 μmol/L bortezomib for 18 h. Cell survival was scored as described in Materials and Methods. *Columns,* mean; *bars,* SD. *, $P < 0.05$, statistically significant deviation from control (GFP) values. *Inset,* Western blot of control LNCaP cells transiently transfected with the constructs expressing GFP and RNAi directed against Bim. GFP-positive and GFP-negative cells were sorted by FACS, lysed, and analyzed for Bim expression by Western blot using GAPDH as a loading control. **B,** bortezomib and TRAIL-mediated apoptosis in APAF-1-deficient MEFs. MEFs were treated as indicated for 18 h and scored for nuclear DAPI uptake as described in Materials and Methods. *Columns,* mean of nuclei and cell counts from two independent experiments; *bars,* SD. *, $P < 0.05$, statistically significant deviation from wild-type value.

well vary with cell type. In any case, these findings and those we reported previously (6) provide the rational for further exploration of the potential of combining bortezomib and TRAIL in cancer therapy.

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