Cancer Therapy: Preclinical

Bortezomib Primes Neuroblastoma Cells for TRAIL-Induced Apoptosis by Linking the Death Receptor to the Mitochondrial Pathway

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Abstract

Purpose: Searching for novel strategies to modulate apoptosis in neuroblastoma, we investigated the potential of the proteasome inhibitor bortezomib.

Experimental Design: The effect of bortezomib on TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis signaling pathways was analyzed in neuroblastoma cell lines, primary neuroblastoma cultures, and in an in vivo model.

Results: Bortezomib synergistically cooperates with TRAIL to induce apoptosis and to reduce colony formation of neuroblastoma cells (combination index: 0.5). Mechanistic studies reveal that bortezomib profoundly enhances TRAIL-induced cleavage of Bid into tBid, accumulation of tBid in the cytosol, and its insertion into mitochondrial membranes, pointing to a concerted effect on Bid cleavage (TRAIL) and stabilization of tBid (bortezomib), which links the death receptor to the mitochondrial pathway. In addition, bortezomib increases expression of p53 and Noxa. All these changes lead to increased activation of Bax and Bak, loss of the mitochondrial membrane potential, cytochrome c release, caspase activation, and caspase-dependent apoptosis on treatment with bortezomib and TRAIL. Knockdown of Bid, Noxa, or p53 significantly delays the kinetic of bortezomib- and TRAIL-induced apoptosis, whereas it does not confer long-term protection. By comparison, overexpression of Bcl-2, which simultaneously antagonizes tBid and p53, significantly inhibits bortezomib- and TRAIL-induced apoptosis and even rescues clonogenic survival. Importantly, bortezomib and TRAIL act in concert to trigger apoptosis and to suppress tumor growth in patient-derived primary neuroblastoma cells and in an in vivo model of neuroblastoma.

Conclusions: Bortezomib represents a promising new approach to prime neuroblastoma cells toward TRAIL, which warrants further investigation.

Clin Cancer Res; 17(10); 1–15. ©2011 AACR.

Introduction

Neuroblastoma is the most frequent pediatric solid tumor outside the central nervous system (1, 2). In contrast to remarkable improvements in the survival and cure rates that have been encountered for many childhood malignancies, the prognosis of children older than 18 months with advanced stage neuroblastoma remains poor with long-term survival rates of less than 40% despite aggressive treatment protocols (3). This underscores the requirement for new treatment strategies.

Apoptosis or programmed cell death plays a crucial role in the regulation of tissue homeostasis (4) and is a critical mediator of therapy-induced cytotoxicity (e.g., in response to chemo- or radiotherapy; ref. 5). Apoptosis pathways are initiated via 2 principal pathways, that is, the death receptor (extrinsic) pathway or the mitochondrial (intrinsic) pathway leading to activation of caspases and caspase-dependent apoptosis (5). The death receptor pathway is typically stimulated by ligation of death receptors of the TNF receptor superfamily resulting in activation of caspase-8 at the death-inducing signaling complex (DISC), which initiates direct cleavage of downstream effector caspases (6). The mitochondrial pathway involves the release of proteins from the mitochondrial intermembrane space into the cytosol such as cytochrome c, triggering caspase-3 activation as a result of the formation of the cytochrome c/Apaf-1/caspase-9 containing apoptosome complex (7). Mitochondrial outer membrane permeabilization is tightly controlled by various factors including the Bcl-2 family of proteins (8). Bcl-2 family proteins consist of both anti-apoptotic members, Bcl-2 and Mcl-1, as well as proapoptotic molecules such as Bax, Bak, and BH3 domain only molecules (e.g., Bid, Bim, and Noxa; ref. 8).

Agents that stimulate TRAIL (TNF-related apoptosis-inducing ligand) receptors, such as recombinant soluble...
Our study provides first evidence that the proteasome inhibitor Bortezomib synergistically induces apoptosis together with TRAIL (TNF-related apoptosis-inducing ligand) in neuroblastoma cells. The clinical relevance is underscored by parallel experiments in patient-derived primary neuroblastoma cells. The concerted action of Bortezomib and TRAIL on cleavage of Bid into tBid, stabilization of tBid, and its insertion into mitochondrial membranes is identified as a novel mechanism of this synergistic interaction, linking death receptor to mitochondrial signaling. Thus, this preclinical evaluation of a rational combination of 2 new classes of targeted drugs identifies a novel indication for Bortezomib in combination with TRAIL in neuroblastoma. Both agents are currently evaluated as monotherapy in early clinical trials against childhood cancer. Our study now provides the molecular basis for the design of future clinical studies of the combination of Bortezomib and TRAIL in neuroblastoma and thus has important clinical implications.

TRAIL or agonistic TRAIL receptor antibodies, present promising experimental cancer therapeutics for cancer cell–selective induction of cell death (6). Results from early clinical trials indicate that TRAIL receptor agonists can be safely administered alone and in combination, for example, with standard chemotherapy (6, 9). However, various human cancers, including neuroblastoma, have developed mechanisms to evade the induction of apoptosis on stimulation with TRAIL (10), calling for the identification and validation of agents that could be used along with TRAIL receptor agonists to enhance the efficacy of TRAIL-based regimens.

There is accumulating evidence that inhibition of the proteasome may provide a means to enhance the sensitivity of cancer cells toward TRAIL (11). Bortezomib (PS-341; VELCADE) is an FDA (Food and Drug Administration)-approved dipeptidyl boronic acid compound that reversibly blocks the proteolytic activity of proteasomes and can modulate multiple signaling pathways via its effect on the proteasome as a central regulator of cellular protein turnover (12). Phase I clinical trials in children with refractory solid tumors or leukemia showed that bortezomib is well tolerated but exerts little activity as monotherapy (13, 14). Similarly, bortezomib showed limited in vivo activity as single agent when it was tested against the solid tumor xenograft panel of the pediatric preclinical testing program (15). In preclinical studies, bortezomib as single agent has been reported to suppress neuroblastoma growth (16–19) including examples of chemoresistance and metastatic disease (20–22). Together, these data indicate that bortezomib represents an interesting investigational agent for the treatment of neuroblastoma. However, the question whether or not bortezomib can be exploited in combination protocols to prime neuroblastoma cells for cell death induction, for example toward the death receptor ligand TRAIL, has not yet been addressed. Therefore, we investigated in the present study the antitumor activity of bortezomib alone and in combination with TRAIL in neuroblastoma.

Materials and Methods

**Cell culture and chemicals**

Neuroblastoma cell lines were obtained from the American Type Culture Collection and maintained in RPMI 1640 or Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc.), supplemented with 10% fetal calf serum (FCS; Biochrom), 1 mmol/L glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 25 mmol/L HEPES (Biochrom) as described previously (23). Genomic characteristics of neuroblastoma cell lines are summarized in Supplementary Figure S1B. N-benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was purchased from Bachem and bortezomib from Jansen-Cilag. TRAIL was obtained from R&D Systems and chemicals were purchased from Sigma unless otherwise indicated.

**Primary cultured neuroblastoma cells**

Primary neuroblastoma cells were isolated by mechanical disaggregation from surgical specimens obtained from patients with stage 4 neuroblastoma and cultured in DMEM supplemented with 1 mmol/L glutamine, 1% penicillin/streptomycin, 25 mmol/L HEPES, and 10% FCS. The study was approved by the Ethics Committee, Medical Faculty, University of Ulm, Ulm, Germany. Neuroblastoma cells were characterized by GD2 synthase mRNA expression using RT-PCR (reverse transcriptase PCR; data not shown). Characteristics of neuroblastoma samples are summarized in Supplementary Table S1.

**Determination of apoptosis, metabolic activity, and clonogenic survival**

Apoptosis was determined by fluorescence-activated cell-sorting (FACScan, BD Biosciences) analysis of DNA fragmentation of propidium iodide (PI)-stained nuclei as described previously (23). The percentage of specific apoptosis was calculated as follows: 100 × [experimental apoptosis (%) – spontaneous apoptosis (%)]/[100% – spontaneous apoptosis (%)]. Cell viability was assessed by MTT assay according to the manufacturer’s instructions (Roche Diagnostics). For clonogenic assay, cells were seeded as single cells (200 cells/well) in 6-well plates for 24 hours, treated for 6 hours before medium was exchanged and recovered for additional 14 days before staining with 0.75% crystal violet, 50% ethanol, 0.25% NaCl, and 1.57% formaldehyde.

**RNA interference**

For transient gene knockdown, cells were seeded at 1.2 × 10^5 per well in a 6-well tissue culture plate and allowed to settle overnight. Cells were transfected with 150 pmol of...
each sequence of Stealth RNAi against Bax and Bak or Bid or nontargeting control siRNA (Invitrogen) using TransMessenger transfection (Qiagen), which was replaced by complete medium after 3.5 hours. After 72 hours of transfection, cells were reseeded in a 24-well tissue culture plate, allowed to settle overnight, and treated with bortezomib and TRAIL.

**Transduction**

For stable gene knockdown, short hairpin RNA (shRNA) targeting p53 sequence (5'-GATCCCCGACTCCAGTGG-TAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGAAAAA-3'; ref. 24) or shRNA targeting Noxa (5'-GATCCCCGACTCCAGTGG-TAATCTACTTCAAGAGAGTAGATTACCACTGGAGTCTTTTTGAAAAA-3'; ref. 25) and a sequence with no corresponding part in the human genome (gatcatgtagatacgctca) that was used as control were cloned into pRETiRO-SUPER as previously described (26). Stable clones were generated by selection with 1 μg/mL puromycin (Clontech). For Bcl-2 overexpression, cells were transduced with pMSCV vector containing mouse Bcl-2 or empty vector using the packaging cell line PT67 (BD Biosciences). Stable cell lines were selected by 10 μg/mL blasticidin (Invitrogen).

**Western blot analysis**

Western blot analysis was conducted as described previously (23) using the following antibodies: mouse anti-Bm, mouse anti–caspase-8, mouse anti-cFLIP, and mouse anti-Noxa (1:1,000; Alexis Biochemicals), rabbit anti-Bcl-XL, mouse anti–FADD, mouse anti-Smac, and mouse anti-XIAP [X-linked inhibitor of apoptosis protein]; clone 28; 1:1,000; BD Transduction Laboratories); rabbit anti-Bak, mouse anti–Bcl-2, rabbit anti–caspase-9, mouse anti–cytochrome c, and mouse anti-p53 (1:1,000; BD Pharmingen); rabbit anti-Bid, rabbit anti–Bim, and rabbit anti–caspase-3 (1:1,000; Cell Signaling); rabbit anti–TRAIL receptor 2 (1:500; Chemicon); goat anti–c-FLAP-1 and rabbit anti–Survivin (1:1,000; R&D Systems); rabbit anti–c-FLAP-2 (1:1,000; Epitomics), mouse anti–OxPhos complex IV (1:2,000; Invitrogen); goat anti–Bik/NBK (1:1,000; Santa Cruz Biotechnology); rabbit anti–Puma (1:500; Sigma); rabbit anti–Mcl-1 (Stressgen); and rabbit anti–BaxNT (1:5,000; Upstate Biotechnology). Mouse anti–α-tubulin (1:3,000; Calbiochem), mouse anti–GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 1:5,000; HyTest), or mouse anti–β-actin (1:10,000; Sigma) were used as loading controls. Goat anti–mouse IgG, donkey anti–goat IgG, and goat anti–rabbit IgG conjugated to horseradish peroxidase (1:5,000; Santa Cruz Biotechnology); and goat anti–mouse IgG1, goat anti–mouse IgG2b or rat anti–mouse kappa (clone 187.1; 1:5,000; Southern Biotech) conjugated to horseradish peroxidase were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience). Representative blots of at least 2 independent experiments are shown. Densitometric analysis was conducted using Image J digital imaging software.

**Cell surface staining**

To determine surface expression of TRAIL receptors, cells were incubated with mouse anti–TRAIL receptor 1 (TRAIL-R1) to -R4 antibodies (10 μg/mL; all from Alexix) for 30 minutes at 4°C, washed in PBS containing 1% FCS, incubated with rabbit anti-mouse F(ab')2; IgG/biotin (5 μg/mL; BD Bioscience) for 20 minutes at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-PE (phycocerythrin; 0.25 μg/mL; BD Bioscience) for 20 minutes at 4°C in the dark, and analyzed by flow cytometry.

**Caspase activity assay**

Caspase-3 activity was determined in living, nonfixed, nonlysed cells using caspase-3 substrate conjugated to rhodamine R110: zVAD.fmk-R110 (zDEVD-R110; Molecular Probes). Cells were incubated with caspase-3 substrate for 30 minutes at 37°C and immediately analyzed by flow cytometry (27).

**TRAIL DISC immunoprecipitation**

Cells were incubated for 30 minutes at 37°C with Flag-tagged TRAIL (1 μg/mL; Alexis) and/or bortezomib or left untreated. Immunoprecipitation of the TRAIL DISC was carried out as previously described (28).

**Determination of Bax and Bak activation**

Bax and Bak activation was determined by immunoprecipitation as previously described (29). Briefly, cells were lysed in CHAPS lysis buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl; 1% CHAPS). An amount of 1 mg protein was incubated with 8 μg mouse anti-Bax antibody (clone 6A7) or 0.5 μg mouse anti-Bak (Ab-1) overnight at 4°C followed by addition of 10 μL pan-mouse IgG Dynabeads, incubated for 2 hours at 4°C, washed with CHAPS lysis buffer, and analyzed by Western blotting using rabbit anti-BaxNT antibody or rabbit anti-Bak antibody.

**Determination of mitochondrial membrane potential and cytochrome c release**

To determine mitochondrial transmembrane potential, potential cells were incubated with CMXRos (1 μmol/L; Molecular Probes) for 30 minutes at 37°C and immediately analyzed by flow cytometry. Cytochrome c release was assessed by flow cytometry as previously described (30). To analyze cytochrome c and Bid in cytosolic and mitochondrial extracts by Western blot, cells were harvested and washed with PBS. Cells were suspended in lysis buffer (2 mmol/L NaH2PO4, 16 mmol/L Na2HPO4, 150 mmol/L NaCl, 500 mmol/L sucrose, 1 mmol/L DTT, Protease Inhibitor cocktail, and 0.5 mg/mL digitonin) for 3 minutes on ice. Unbroken cells, mitochondria, and nuclei were removed by centrifugation at 14,000 rpm for 1 minute at 4°C. The supernatant was collected as cytosolic fraction and the pellet was resuspended in lysis buffer [30 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X, 10% glycerol, protease inhibitor cocktail, 2 mmol/L DTT, and 500 μmol/L...
phenylmethylsulfonylfluoride (PMSF) for 2 hours at 4°C and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supematant was collected as mitochondrial fraction. Protein expressions of cytochrome c or Bid were analyzed by Western blotting. OxPhos complex IV and α-tubulin were used to control the purity and loading of the mitochondrial and cytosolic fractions.

Chorioallantoic membrane assay

Chorioallantoic membrane (CAM) assay was done as described previously (30). Briefly, 2 × 10^6 cells were resuspended in 10 μL serum-free medium and 10 μL Matrigel matrix (BD Biosciences) and implanted on the CAM of fertilized chicken eggs on day 8 of incubation. On day 10, tumors were treated with 15 ng/mL TRAIL and 12.5 μmol/L bortezomib alone or in combination daily for 2 days. Four days after seeding, tumors were sampled with the surrounding CAM, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5-μm sections, and analyzed by immunohistochemistry using 1:1 hematoxylin and 0.5% eosin. Images were digitally recorded at a magnification of 2× with an AX70 microscope (Olympus) and tumor areas were analyzed with ImageJ digital imaging software.

Statistical analysis

Statistical significance was assessed by Student’s t tests (2-tailed distribution, 2-sample, unequal variance). Interaction between bortezomib and TRAIL was analyzed by the combination index (CI) method on the basis of that described by Chou (31) using CalcuSyn software (Biosoft). CI < 0.9 indicates synergism, 0.9–1.1, additivity, and >1.1 antagonism.

Results

Bortezomib sensitizes neuroblastoma cells for TRAIL-induced apoptosis

To investigate the therapeutic potential of proteasome inhibition in the regulation of TRAIL-induced apoptosis in human neuroblastoma, we selected SH-EP, SK-N-AS, and

![Figure 1. Bortezomib sensitizes neuroblastoma cells for TRAIL-induced apoptosis.](image)
LAN-5 cells from a panel of neuroblastoma cell lines, as they all express caspase-8, a key component of the death receptor pathway (Supplementary Fig. S1A). Analysis of different administration schedules revealed that pretreatment for 4 hours with the proteasome inhibitor bortezomib before the addition of TRAIL was superior to prime neuroblastoma cells to TRAIL-induced apoptosis compared with co- or posttreatment with bortezomib or to another time of prestimulation with bortezomib (Supplementary Fig. S2A–C). Therefore, we used a 4-hour pretreatment period in all subsequent experiments. Preexposure to bortezomib acted in a highly synergistic manner with TRAIL to reduce cell viability in several neuroblastoma cell lines as shown by calculation of CI (Fig. 1A and Supplementary Fig. S2D). Also, bortezomib profoundly enhanced TRAIL-induced apoptosis in a dose- and time-dependent manner (Fig. 1B and C). Besides recombinant soluble TRAIL, Bortezomib acted in concert with agonistic TRAIL receptor antibodies to reduce viability and to trigger apoptosis (data not shown). Importantly, bortezomib cooperated with TRAIL to suppress colony formation of neuroblastoma cells compared with either treatment alone (Fig. 1D), showing an effect on long-term survival. Moreover, we investigated whether bortezomib overcomes resistance to TRAIL in neuroblastoma cells with caspase-8 silencing due to epigenetic inactivation (32). Although bortezomib failed to sensitize neuroblastoma cells toward TRAIL that do not express caspase-8, pretreatment with IFNγ upregulated caspase-8 expression and significantly increased TRAIL-induced apoptosis (Fig. 1E). Together, this set of
experiments shows that bortezomib primes neuroblastoma cells for TRAIL-induced apoptosis resulting in suppression of clonogenic survival.

**Activation of Bid and Bax/Bak contributes to bortezomib-mediated sensitization to TRAIL.**

To elucidate the molecular mechanisms underlying the cooperative cytotoxicity of bortezomib and TRAIL, we monitored expression levels of a panel of apoptosis regulatory proteins. Bortezomib caused a marked increase in protein expression of Noxa, p53, and Mcl-1, a slight accumulation of BimEL, cIAP-1/2, and cFLIPS expression and upregulation of TRAIL-R2 surface expression (Fig. 2A). Analysis of the DISC that forms on stimulation with TRAIL revealed that bortezomib only slightly enhanced the recruitment of caspase-8 and FADD into the DISC on TRAIL receptor ligation, whereas it substantially increased the TRAIL-stimulated recruitment of cFLIPS (Fig. 2B). This indicates that the net result of bortezomib-mediated upregulation of TRAIL-R2 and cFLIPS is only a minor increase in caspase-8 at the TRAIL DISC and therefore unlikely the key mechanism for apoptosis sensitization.

Monitoring of caspase activation showed that bortezomib profoundly enhanced TRAIL-induced cleavage of caspase-8, -3, and -9 into active fragments accompanied by an increased proteolytic turnover of the caspase proenzymes (Fig. 2C), increased caspase-3 activity (Fig. 2D), and cleavage of Bid into tBid (Fig. 2C). The broad-range caspase inhibitor zVAD.fmk blocked bortezomib- and TRAIL-induced apoptosis (Fig. 2E), including NLF neuroblastoma cells, after restoration of caspase-8 expression by pretreatment with IFNγ (Supplementary Fig. S3), showing caspase dependency.

Because tBid links the death receptor to the mitochondrial apoptosis pathway, we reasoned that proteasome inhibition by bortezomib might prevent the degradation of tBid that is newly generated on treatment with TRAIL, thereby causing its accumulation. To test this hypothesis, we treated cells with TRAIL to trigger cleavage of Bid into tBid. Then, TRAIL was removed, zVAD.fmk was added to inhibit further caspase-mediated Bid cleavage, and tBid levels were monitored in the presence or absence of bortezomib. Importantly, the addition of bortezomib delayed the decline of tBid (Fig. 3A), indicating that bortezomib

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### Figure 2. Bortezomib modulates expression of pro- and antiapoptotic proteins and sensitizes for TRAIL-induced caspase activation.

A, SH-EP cells were treated with 50 nmol/L Bortezomib for 10 hours. Protein expression of pro- and antiapoptotic proteins was assessed by Western blotting (left, middle); β-actin, GAPDH, and α-tubulin served as loading controls. D425 and Jurkat cell lysates were used as positive controls for Bmf and Bik detection, respectively (pc). Right, surface expression of TRAIL receptors was determined by flow cytometry after SH-EP cells were treated (bold line) or not (thin line) with 50 nmol/L Bortezomib for 10 hours. Anti-IgG antibody was used as control (dotted line). Results from 1 representative experiment are shown. B, SH-EP cells were pretreated or not with 50 nmol/L Bortezomib (B) for 4 hours and then treated with Flag-tagged TRAIL (T) for 30 minutes. The DISC was precipitated with anti-Flag M2 antibody. Recruitment of caspase-8, FADD, cFLIPS, and cFLIPL was analyzed by Western blotting as well as TRAIL-R2 expression (left); β-actin was used as loading control in the corresponding lysates (right). hc, heavy chain.

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### Table 2

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**Figure 3. Bortezomib delays the decline of tBid.**

Importantly, the addition of bortezomib delayed the decline of tBid (Fig. 3A), indicating that bortezomib...
interferes with proteasomal degradation of Bid. Because tBid translocates to mitochondrial membranes to trigger mitochondrial outer membrane permeabilization, we also analyzed tBid in the mitochondrial fraction. Interestingly, the combination of bortezomib and TRAIL caused a profound increase of tBid in mitochondrial membranes (Fig. 3B, lane 4). To test whether Bid is required for apoptosis induction, we silenced Bid by RNA interference (RNAi; Fig. 3C). Importantly, knockdown of Bid significantly delayed the kinetic of bortezomib- and TRAIL-
Figure 4. Bortezomib-mediated accumulation of Noxa and p53 determines the kinetics of apoptosis induction. A, SH-EP cells were transduced with a vector containing Noxa or control shRNA. Knockdown of Noxa expression was controlled by Western blotting; β-actin served as loading control (left). Transduced cells were treated with 50 nmol/L Bortezomib (B) and/or 1.25 ng/mL TRAIL (T) for indicated times; treatment with camptothecin for 24 hours served as positive control (CPT) (right). B-D, Knockdown of Bak or Bax by shRNA was confirmed by Western blotting; β-actin served as a loading control (left). Transduced cells were treated with 50 nmol/L Bortezomib (B) and/or 1.25 ng/mL TRAIL (T) for indicated times; treatment with camptothecin for 24 hours served as positive control (CPT) (right).
induced apoptosis, whereas it did not confer protection on prolonged exposure to both compounds (Fig. 3C). The analysis of mitochondrial outer membrane permeabilization revealed that bortezomib significantly enhanced TRAIL-induced loss of mitochondrial membrane potential (MMP) and cytochrome c release in a time-dependent manner compared with treatment with TRAIL alone (Fig. 3D).

Because expression levels of Bax and Bak were not altered by bortezomib (Fig. 2A) and Bax and Bak are activated by conformational changes, we carried out immunoprecipitation with conformation-specific antibodies. Bortezomib and TRAIL acted in concert to trigger Bax and Bak activation (Fig. 3E). Notably, silencing of Bax and Bak delayed bortezomib- and TRAIL-induced apoptosis (Fig. 3F). Together, these experiments show that Bid, Bax, and Bak contribute to bortezomib-mediated sensitization to TRAIL.

**Bortezomib-mediated accumulation of Noxa and p53 determines the kinetics of apoptosis induction**

To explore the functional relevance of bortezomib-mediated accumulation of Noxa and p53, we silenced their expression by RNAi. Knockdown of Noxa significantly reduced bortezomib- and TRAIL-triggered apoptosis at an early time point (6 hours), whereas it failed to block apoptosis on longer treatment (Fig. 4A). Interestingly, silencing of Noxa delayed activation of Bak, but not of Bax (Fig. 4B), in line with predominant binding of Noxa to Mcl-1, which in turn primarily sequesters Bak (33). Similarly, silencing of p53 significantly reduced bortezomib- and TRAIL-triggered apoptosis at early time points, whereas it did not confer protection on longer exposure for 24 or 48 hours (Fig. 4C). Consistently, kinetics of both Bax and Bak activation were delayed on silencing of p53 but similarly occurred on longer exposure to bortezomib and TRAIL (Fig. 4D). Interestingly, knockdown of p53 did not prevent bortezomib-stimulated Noxa expression, whereas it reduced constitutive expression of Bax and Bak (Fig. 4C, left), pointing to p53-independent accumulation of Noxa by bortezomib. Camptothecin was used as positive control to confirm that knockdown of p53 or Noxa protects against DNA damage–induced apoptosis (Fig. 4A and C). This set of experiments shows that silencing of either Noxa or p53 delays the kinetic of apoptosis induction by bortezomib and TRAIL.

**Overexpression of Bcl-2 inhibits synergistic induction of apoptosis by Bortezomib and TRAIL**

To further investigate the requirement of the mitochondrial pathway for the synergistic interaction of bortezomib and TRAIL, we ectopically expressed Bcl-2. Importantly, Bcl-2 overexpression significantly reduced apoptosis even on prolonged treatment with bortezomib and TRAIL for 48 hours (Fig. 5A) and significantly rescued the reduction in colony formation triggered by bortezomib and TRAIL (Fig. 5B), showing that Bcl-2 conferred long-term protection. Mechanistic studies revealed that ectopic expression of Bcl-2 substantially reduced cleavage of Bid into tBid, insertion of tBid into mitochondrial membranes, loss of MMP as well as cytochrome c release (Fig. 5C and D), whereas the combination treatment had no effect on expression levels of ectopically expressed Bcl-2 (Supplementary Fig. S4). These experiments underscore that mitochondrial outer membrane permeabilization is required for the synergistic induction of apoptosis by bortezomib and TRAIL and point to a Bcl-2–controlled mitochondrial feedback amplification loop to tBid that is probably mediated by caspases.

**Bortezomib cooperates with TRAIL to induce apoptosis in primary neuroblastoma cells and to suppress neuroblastoma growth in vivo**

To evaluate the clinical relevance of our approach, we carried out experiments with primary neuroblastoma cultures, which were established from surgical samples and characterized by GD2 synthase expression (data not shown). Intriguingly, pretreatment with bortezomib profoundly increased TRAIL-induced apoptosis in primary neuroblastoma cells obtained from 6 different specimens, which were resistant to TRAIL in the absence of bortezomib (Fig. 6A). Induction of apoptosis was accompanied by markedly enhanced caspase-3 activation (Fig. 6B).

Finally, to test the antitumor activity of bortezomib and TRAIL in vivo, we used the CAM assay, an established in vivo tumor model, for example, for neuroblastoma (29, 30, 34, 35). Neuroblastoma cells were seeded on the CAM of chicken embryos, allowed to settle and to initiate tumors followed by local treatment with TRAIL in the presence or absence of bortezomib. Importantly, bortezomib and TRAIL acted in concert to significantly suppress tumor growth of neuroblastoma in vivo compared with either agent
alone (Fig. 6C). These findings show that bortezomib and TRAIL cooperate to induce apoptosis in primary neuroblastoma cells and to suppress neuroblastoma growth in vivo.

Discussion

In the present study, we investigated the potential of the proteasome inhibitor bortezomib to modulate apoptosis sensitivity of neuroblastoma. Here, we provide the first evidence that bortezomib presents a powerful tool to prime neuroblastoma cells for TRAIL-induced apoptosis in vitro and in vivo. Bortezomib synergistically interacts with TRAIL to trigger apoptosis, as shown by CI, and to suppress clonogenic growth. Previously, bortezomib was investigated as single agent in neuroblastoma and shown to suppress tumor growth (16–22). However, the question whether bortezomib can be used in combination protocols to lower the threshold for apoptosis induction has not yet been answered. The issue of developing new synergistic combination therapies for neuroblastoma is important, as neuroblastoma cells have developed multiple mechanisms to evade apoptosis.

We identify as a novel molecular mechanism the concerted action of bortezomib and TRAIL on cleavage of Bid into tBid, stabilization of tBid, which is prone to ubiquitin-mediated proteasomal degradation (36), and its insertion into mitochondrial membranes (Fig. 6D). Thus, bortezomib links the death receptor pathway to mitochondrial outer membrane permeabilization, which may in particular be relevant in certain cell types such as type II cells that depend on the mitochondrial pathway for TRAIL-induced apoptosis (37).

Furthermore, our study is the first demonstration that Noxa becomes dispensable for apoptosis on prolonged exposure to bortezomib and TRAIL, although it determines the initial kinetic of apoptosis induction. Although Noxa
has been reported to be required for apoptosis following monotherapy with bortezomib (38–40), its contribution to apoptosis on combined treatment with bortezomib and TRAIL has not yet been explored. Thus, the role of Noxa in apoptosis may differ for single-agent versus combination therapy with bortezomib. Noxa may exert its proapoptotic function by opposing the antiapoptotic effect of Mcl-1, which is also upregulated by bortezomib, and by targeting Mcl-1 for proteasomal degradation (41). In addition, caspase-mediated cleavage of Mcl-1 yielding a C-terminal proapoptotic fragment may neutralize its accumulation by bortezomib (42, 43). Similarly, p53 determines the kinetic of apoptosis induction in response to bortezomib and TRAIL without affecting long-term survival. This likely involves both transcriptional and posttranscriptional events, as constitutive Bax and Bak expression as well as bortezomib and TRAIL-induced Bax/Bak activation are reduced on p53 silencing. Although the sensitivity to bortezomib has been associated with wild-type p53 status in a recent study (44),

Figure 6. Bortezomib cooperates with TRAIL to induce apoptosis in primary neuroblastoma cells and to suppress neuroblastoma growth in vivo. A, primary cultured neuroblastoma cells (NB) were treated for 24 hours with 50 nmol/L Bortezomib and/or 100 ng/mL TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of PI-stained nuclei and percentage of apoptosis is shown. Data represent mean ± SEM of at least 2 independent experiments carried out in triplicate. B, primary cultured neuroblastoma cells (NB16) were treated with 50 nmol/L Bortezomib (B) and/or 100 ng/mL TRAIL (T) for 24 hours. Expression of caspase-3 was assessed by Western blot analysis. β-Actin was used as loading control (right). Cleavage fragments are indicated by arrows. C, SH-EP cells were seeded on the CAM of chicken embryos. On day 10, tumors were treated with 15 ng/mL TRAIL and 12.5 μmol/L Bortezomib alone or in combination daily for 2 days. Tumor growth was analyzed using hematoxylin and eosin–stained paraffin sections of the CAM as described in Materials and Methods. Shown is the tumor area, error bars indicate mean ± SD of 6 samples per group; *, P < 0.05. Similar results were obtained in 2 independent experiments. D, scheme of synergistic interaction of TRAIL and Bortezomib in neuroblastoma cells. Bortezomib increases TRAIL-induced Bid cleavage into tBid, its stabilization and accumulation in mitochondrial membranes, enhances protein expression of p53 and Noxa, mitochondrial outer membrane permeabilization, and caspase-dependent apoptosis. Bcl-2 overexpression inhibits mitochondrial outer membrane permeabilization, caspase activation, and apoptosis. Bortezomib-mediated increase in surface expression of TRAIL-R2 and cFLIP results in only slightly enhanced recruitment of caspase-8 to the TRAIL DISC. See text for more details.
bortezomib-induced apoptosis was reported to occur in a p53-independent manner in 2 other reports (45, 46), pointing to a context-dependent role of p53 in bortezomib-induced apoptosis. Of note, we found increased expression of Noxa on treatment with bortezomib independently of p53, pointing to posttranscriptional mechanisms. The contribution of p53 to the initial kinetics of apoptosis induction is interesting in light of recent evidence that the p53/MDM2/p14ARF pathway is frequently altered in relapsed neuroblastoma (47).

Although knockdown of Bid, Noxa, or p53 delays the kinetic of cell death, thereby underscoring their involvement in apoptosis induction, it is insufficient to rescue survival on prolonged exposure to bortezomib and TRAIL. By comparison, overexpression of Bcl-2, which simultaneously antagonizes tBid and p53, is able to provide long-term protection. This suggests that several proapoptotic proteins, such as tBid, p53, and Noxa, contribute to bortezomib and TRAIL-triggered apoptosis. By comparison, proteasome inhibitors and TRAIL have previously been reported to trigger apoptosis also in Bcl-2-overexpressing leukemia cells (48), pointing to a context-dependent involvement of Bcl-2.

Compared with the key role of the mitochondrial pathway in the bortezomib-mediated sensitization to TRAIL, our data suggest that modulation of death receptor signaling by bortezomib plays a minor role. While we found increased surface expression of TRAIL-R2 on exposure to bortezomib in line with previous studies (49–51), cFLIP, simultaneously accumulates and is also recruited into the TRAIL DISC on ligation of TRAIL receptors. As a net result, there is only a minor increase in the recruitment of caspase-8 into the TRAIL DISC, indicating that the bortezomib-mediated increase in TRAIL-R2 surface expression unlikely accounts for the bortezomib-mediated sensitization to TRAIL-induced apoptosis.

Our study has several important implications. First and foremost, it provides the rational for future (pre)clinical evaluation of the combination strategy with bortezomib and TRAIL. The clinical relevance of our findings is underscored by concomitant experiments in primary neuroblastoma tumor samples. Furthermore, experiments in an in vivo model of neuroblastoma similarly show the cooperative antitumor activity of bortezomib and TRAIL. Because both bortezomib and TRAIL receptor agonists are currently evaluated as single agents in early clinical trials against childhood cancer (refs. 13, 14; www.clinicaltrials.org), it is feasible that this approach can be translated into clinical application in pediatric oncology. It is interesting to note that bortezomib showed no single-agent antitumor activity in initial clinical trials with children (13, 14) or when it was tested against the solid tumor xenograft panel of the pediatric preclinical testing program (15). Also, monotherapy with mapatumumab, a TRAIL-R1 agonistic monoclonal antibody, showed limited in vitro and in vivo activity when tested by the pediatric preclinical testing program (52) underlining that bortezomib-based combination strategies, for example in combination with TRAIL, may be of particular interest in neuroblastoma. Of note, in neuroblastoma with epigenetic silencing of caspase-8, which make up to 75% of neuroblastoma cases (53), the combination approach of bortezomib and TRAIL may also be applicable to trigger apoptosis after restoration of caspase-8 expression (e.g., by IFNγ).

Second, from a mechanistic point of view, our data provide novel insights into the signaling pathways that are regulated by the combination of bortezomib and TRAIL in cancer cells and that can be exploited to achieve synergistic antitumor activities. Thus, beyond the potential application of our findings to neuroblastoma, our results are also relevant from a more general standpoint in other types of cancer for the development of bortezomib-based combination regimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

The authors thank C.A. Schmitt (Berlin, Germany) for providing mouse Bcl-2 vector.

Grant Support

This work has been partially supported by grants from the Deutsche Forschungsgemeinschaft, Wilhelm Sander-Stiftung, European Community, (ApogTrain, APO-SYS) and IAP/6-18 (to S. Fulda).

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Received September 13, 2010; revised February 22, 2011; accepted March 10, 2011; published OnlineFirst April 1, 2011.

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Clin Cancer Res  Published OnlineFirst April 1, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-10-2451
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