Bortezomib primes neuroblastoma cells for TRAIL-induced apoptosis by linking the death receptor to the mitochondrial pathway

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Translational Relevance

Our study provides first evidence that the proteasome inhibitor Bortezomib synergistically induces apoptosis together with TRAIL in neuroblastoma cells. The clinical relevance is underscored by parallel experiments in patients’ 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 two 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.
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-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. Additionally, 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 upon treatment with Bortezomib and TRAIL. Knockdown of either Bid, Noxa or p53 significantly delays the kinetic of Bortezomib- and TRAIL-induced apoptosis, whereas it does not confer longterm 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 patients’ derived primary neuroblastoma cells and in an in vivo model of neuroblastoma.

Conclusions: Bortezomib represents a promising new approach to prime neuroblastoma cells towards TRAIL, which warrants further investigation.
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 longterm 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, for example in response to chemo- or radiotherapy (5). Apoptosis pathways are initiated via two principal pathways, i.e. 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 tumor necrosis factor (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, e.g. Bcl-2 and Mcl-1, as well as pro-apoptotic molecules such as Bax, Bak and BH3 domain only molecules, e.g. Bid, Bim and Noxa (8).

Agents that stimulate TRAIL receptors such as recombinant soluble TRAIL or agonistic TRAIL receptor antibodies present promising experimental cancer
therapeutics for cancer cell selective induction of cell death (6). Results from early clinical trial 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 upon 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 mean to enhance the sensitivity of cancer cells towards TRAIL (11). Bortezomib (PS-341, VELCADE), is a FDA-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 demonstrated 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 towards 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 (Manassas, VA, USA) and maintained in RPMI 1640 or DMEM medium (Life Technologies, Inc., Eggenstein, Germany), supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 mM glutamine (Invitrogen, Karlsruhe, Germany), 1% penicillin/streptomycin (Invitrogen) and 25mM HEPES (Biochrom) as described previously (23). Genomic characteristics of neuroblastoma cell lines are summarized in Suppl. Fig. 1B. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was purchased from Bachem (Heidelberg, Germany), Bortezomib from Jansen-Cilag (Lörrach, Germany). TRAIL was obtained from R&D Systems (Wiesbaden, Germany) and chemicals were purchased by Sigma (Deisenhofen, Germany) 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 mM glutamine, 1% penicillin/streptomycin, 25 mM HEPES and 10% FCS. The study was approved by the Ethics Committee, Medical Faculty, University of Ulm. Neuroblastoma cells were characterized by GD2 synthase mRNA expression using RT-PCR (data not shown). Characteristics of neuroblastoma samples are summarized in Suppl. Tab. 1.

Determination of apoptosis, metabolic activity and clonogenic survival

Apoptosis was determined by fluorescence-activated cell-sorting (FACScan, BD Biosciences, Heidelberg, Germany) analysis of DNA fragmentation of propidium
iodide-stained nuclei as described previously (23). The percentage of specific apoptosis was calculated as follows: 100 x [experimental apoptosis (%) – spontaneous apoptosis (%)]/[100% - spontaneous apoptosis (%)]. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 24h, treated for 6h 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 x 10^5/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 non-targeting control siRNA (Invitrogen) using TransMessenger transfection (Qiagen, Hilden, Germany), which was replaced by complete medium after 3.5h. 72h after transfection cells were re-seeded in a 24-well tissue culture plate, allowed to settle overnight and treated with Bortezomib and TRAIL.

**Transduction**

For stable gene knockdown, shRNA targeting p53 sequence (5´ GATCCCCGACTCCAGTGGTAATCTACTTCAAGAGAGTAGATTACCACTGGAGTC TTTTGGAAAA 3´ (24) or shRNA targeting Noxa (5´ GATCCCCGTAATTATTGACACATTTCTTCAAGAGAAATGTGTCAATAATTACTT TTTGGAAAA 3´ (25) and a sequence with no corresponding part in the human genome (GATCATGTAGATACGCTCA) that was used as control were cloned into
pRETRO-SUPER as previously described (26). Stable clones were generated by selection with 1 μg/ml puromycin (Clontech, Heidelberg, Germany). 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 performed as described previously (23) using the following antibodies: mouse anti-Bmf, mouse anti-caspase-8, mouse anti-cFLIP and mouse anti-Noxa (1:1000; Alexis Biochemicals, Grünberg, Germany), rabbit anti-Bcl-X<sub>L</sub>, mouse anti-FADD, mouse anti-Smac and mouse anti-XIAP (clone 28) (1:1000; BD Transduction Laboratories, Heidelberg, Germany), rabbit anti-Bak, mouse anti-Bcl-2, rabbit anti-caspase-9, mouse anti-cytochrome c and mouse anti-p53 (1:1000; BD Pharmingen, San Diego, CA, USA), rabbit anti-Bid, rabbit anti-Bim and rabbit anti-caspase-3 (1:1000, Cell Signaling, Beverly, MA, USA), rabbit anti-TRAIL receptor 2 (1:500; Chemicon, Billerica, MA, USA), goat anti-clAP-1 and rabbit anti-Survivin (1:1000; R&D Systems), rabbit anti-clAP-2 (1:1000; Epitomics, Burlingname, CA, USA), mouse anti-OxPhos Complex IV (1:2000; Invitrogen), goat anti-Bik/NBK (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Puma (1:500; Sigma), rabbit anti-Mcl-1 (Stressgen, Ann Arbor, MI, USA), rabbit anti-BaxNT (1:5000; Upstate Biotechnology, Lake Placid, NY, USA). Mouse anti-α-tubulin (1:3000; Calbiochem, San Diego, CA), mouse anti-GAPDH (1:5000; HyTest, Turku, Finland) or mouse anti-β-actin (1:10000; Sigma) were used as loading controls. Goat anti-mouse IgG, donkey anti-goat IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Santa Cruz Biotechnology) and goat anti-mouse IgG1, goat anti-mouse IgG2b or rat anti-mouse kappa (clone 187.1) (1:5000; Southern Biotech,
Birmingham, AL, USA) conjugated to horseradish peroxidase were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Bioscience, Freiburg, Germany). Representative blots of at least two independent experiments are shown. Densitometric analysis was performed using Image J digital imaging software.

**Cell surface staining**

To determine surface expression of TRAIL receptors, cells were incubated with mouse anti-TRAIL-R1 to -R4 antibodies (10 µg/ml; all from Alexis) 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 (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: N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone-R110 (zDEVD-R110, Molecular Probes, Karlsruhe, Germany). 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 either with Flag-tagged TRAIL (1 µg/ml; Alexis) and/or Bortezomib or left untreated. Immunoprecipitation of the TRAIL DISC was performed 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 mM HEPES pH 7.4; 150 mM NaCl; 1% CHAPS). 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 10µl pan-mouse IgG Dynabeads, incubated for 2h at 4°C, washed with CHAPS lysis buffer and were 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, cells were incubated with CMXRos (1 µM; Molecular Probes) for 30 minutes at 37°C and immediately analyzed by flow cytometry. Cytochrome c released 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 mM NaH$_2$PO$_4$, 16 mM Na$_2$HPO$_4$, 150 mM NaCl, 500 mM sucrose, 1 mM DTT, Protease Inhibitor cocktail, 0.5 mg/ml digitonin) for 3 minutes on ice. Unbroken cells, mitochondria and nuclei were removed by centrifugation at 14000 rpm for 1 minute at 4°C. The supernatant was collected as cytosolic fraction and the pellet was resuspended in lysis buffer (30 mM Tris HCl, 150 mM NaCl, 1% Triton X, 10% glycerol, Protease Inhibitor cocktail, 2 mM DTT, 500 µM Phenylmethylsulfonylfluoride (PMSF)) for 2h at 4°C and centrifuged at 14000 rpm for 20 minutes at 4°C. The supernatant 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 (CAM) assay

CAM assay was done as described previously (30). Briefly, 2x 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 µM Bortezomib alone or in combination daily for two 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 hematoxyline and 0.5% eosin. Images were digitally recorded at a magnification of 2x with an AX70 microscope (Olympus, Center Valley, PA), tumor areas were analyzed with ImageJ digital imaging software.

Statistical analysis

Statistical significance was assessed by Student's t-Tests (two-tailed distribution, two-sample, unequal variance). Interaction between Bortezomib and TRAIL was analyzed by the Combination index (CI) method based on that described by Chou (31) using CalcuSyn software (Biosoft, Cambridge, UK). Combination index (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 LAN-5 cells from a panel of neuroblastoma cell lines, since they all express caspase-8, a key component of the death receptor pathway (suppl. Fig. 1A). Analysis of different administration schedules revealed that pretreatment for 4h with the proteasome inhibitor Bortezomib before the addition of TRAIL was superior to prime neuroblastoma cells to TRAIL-induced apoptosis compared to co- or posttreatment with Bortezomib or to another time of prestimulation with Bortezomib (suppl. Fig. 2A-C). Therefore, we used a 4h 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 demonstrated by calculation of combination index (Fig. 1A, suppl. Fig. 2D). Also, Bortezomib profoundly enhanced TRAIL-induced apoptosis in a dose- and time-dependent manner (Fig. 1B, 1C). 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 to either treatment alone (Fig. 1D), demonstrating an effect on longterm survival. Moreover, we investigated whether Bortezomib overcomes resistance to TRAIL in neuroblastoma cells with caspase-8 silencing due to epigenetic inactivation (32). While Bortezomib failed to sensitize neuroblastoma cells towards TRAIL that do not express caspase-8, pre-treatment with IFNγ upregulated caspase-8 expression and significantly increased TRAIL-induced apoptosis (Fig. 1E). Together, this set of experiments demonstrates
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, cIAP1/2 and cFLIPS expression and upregulation of TRAIL receptor 2 (TRAIL-R2) surface expression (Fig. 2A). Analysis of the death inducing signaling complex (DISC) that forms upon stimulation with TRAIL revealed that Bortezomib only slightly enhanced the recruitment of caspase-8 and FADD into the DISC upon 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 (Suppl. Fig. 3), demonstrating caspase-dependency.

Since 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 upon 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 interferes with proteasomal degradation of tBid. Since 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 (Fig. 3C). Importantly, knockdown of Bid significantly delayed the kinetic of Bortezomib- and TRAIL-induced apoptosis, while it did not confer protection upon 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 and cytochrome c release in a time-dependent manner compared to treatment with TRAIL alone (Fig. 3D).

Since expression levels of Bax and Bak were not altered by Bortezomib (Fig. 2A) and since Bax and Bak are activated by conformational changes, we performed 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 demonstrate 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 (6h), while it failed to block apoptosis upon 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, while it did not confer protection upon longer exposure for 24h or 48h (Fig. 4C). Consistently, kinetics of both Bax and Bak activation were delayed upon silencing of p53, but similarly occurred upon longer exposure to Bortezomib and TRAIL (Fig. 4D). Interestingly, knockdown of p53 did not prevent Bortezomib-stimulated Noxa expression, while it reduced constitutive expression of Bax and Bak (Fig. 4C, left panel), 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, 4C). This set of experiments demonstrates 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 upon prolonged treatment with Bortezomib and TRAIL for 48h (Fig. 5A) and significantly rescued the reduction
in colony formation triggered by Bortezomib and TRAIL (Fig. 5B), demonstrating that Bcl-2 conferred longterm 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 mitochondrial membrane potential as well as cytochrome c release (Fig. 5C, 5D), while the combination treatment had no effect on expression levels of ectopically expressed Bcl-2 (Suppl. Fig. 4). 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 performed 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 six 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 chorioallantoic membrane (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 to either agent alone (Figure 6C). These findings demonstrate 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 demonstrated by combination index, 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, since 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 upon prolonged exposure to Bortezomib and TRAIL, although it determines the initial kinetic of apoptosis induction. While Noxa has been reported to be required for apoptosis following monotherapy with Bortezomib (38-40), its contribution to apoptosis upon 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 longterm 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 upon p53 silencing. While the sensitivity to Bortezomib has been associated with wild-type p53 status in a recent study (44), Bortezomib-induced apoptosis was reported to occur in a p53-independent manner in two other reports (45, 46), pointing to a context-dependent role of p53 in Bortezomib-induced apoptosis. Of note, we found increased expression of Noxa upon 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).

While knockdown of either Bid, Noxa or p53 delays the kinetic of cell death, thereby underscoring their involvement in apoptosis induction, it is insufficient to rescue survival upon prolonged exposure to Bortezomib and TRAIL. By comparison, overexpression of Bcl-2, which simultaneously antagonizes tBid and p53, is able to provide longterm 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-dependant involvement of Bcl-2.
Compared to 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 upon exposure to Bortezomib in line with previous studies (49-51), cFLIPs simultaneously accumulates and is also recruited into the TRAIL DISC upon 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 demonstrate the cooperative antitumor activity of Bortezomib and TRAIL. Since both Bortezomib and TRAIL receptor agonists are currently evaluated as single agents in early clinical trials against childhood cancer (13, 14) (www.clintrials.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, demonstrated 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.

Conflict of interest: Authors declare that they have no conflict of interest.

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References

Figure legends

Figure 1. Bortezomib sensitizes neuroblastoma cells for TRAIL-induced apoptosis.

Neuroblastoma cells (SH-EP, SK-N-AS, Lan-5) were pretreated (black symbols) or not (white symbols) with 50 nM Bortezomib for 4 h followed by treatment of TRAIL at indicated concentrations.

A, Cell viability was determined by MTT assay after 24h treatment with TRAIL.

B, Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei after 24h treatment with TRAIL and percentage of specific apoptosis is shown.

C, SH-EP neuroblastoma cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL alone or in combination for indicated times. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei and percentage of specific apoptosis is shown.

D, SH-EP cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL for 6 h. Colonies were stained with crystal violet after 14 days and were counted under the microscope. One representative experiment (left panel) and the percentage of colony numbers compared to the untreated control (right panel) are shown.

E, SH-SY5Y (left panel) and Kelly (right panel) cells were treated with 500 U/ml IFNγ for 48h. Re-expression of caspase-8 was assessed by Western blotting (upper panel); β-actin was used as loading control. Cells were treated (black bars) or not (white bars) with 500 U/ml IFNγ for 48h then additionally with Bortezomib (Kelly: 3 nM, SH-SY5Y: 10 nM) for 8h before adding 50 ng/ml TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei after 24h (SH-SY5Y) or 48h (Kelly) treatment with TRAIL and the percentage of specific apoptosis is shown.
In A-C and D, mean + SEM of 3 independent experiments performed in triplicate are shown; in E, mean + SEM of at least 2 independent experiments performed in triplicate are shown. *p<0.05, **p<0.0001.

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

A, SH-EP cells were treated with 50 nM Bortezomib for 10h. Protein expression of pro- and anti-apoptotic proteins were assessed by Western blotting (left, middle panel); β-actin, GAPDH and α-tubulin served as loading control. D425 and Jurkat cell lysates were used as positive controls for Bmf and Bik detection, respectively (pc). In the right panel, surface expression of TRAIL-receptors was determined by flow cytometry after SH-EP cells were treated (bold line) or not (thin line) with 50 nM Bortezomib for 10h. Anti-IgG antibody was used as control (dotted line). Results from one representative experiment are shown.

B, SH-EP cells were pretreated or not with 50 nM Bortezomib (B) for 4h and then treated with FLAG-tagged TRAIL (T) for 30 minutes. The death-inducing signaling complex (DISC) was precipitated with anti-FLAG M2 antibody. Recruitment of caspase-8, FADD, cFLIP_L and cFLIP_S was analyzed by Western blotting as well as TRAIL-receptor 2 expression (left panel); β-actin was used as loading control in the corresponding lysates (right panel). hc, heavy chain.

C, SH-EP cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL at indicated times (hours). Protein expression of caspase-8, -9, -3, Bid and β-actin was assessed by Western blot analysis. Cleavage fragments are indicated by arrows.

D, SH-EP cells were treated either with 50 nM Bortezomib (grey symbols) or 1.25 ng/ml TRAIL (white symbols) alone or in combination (black symbols) at indicated times. Caspase-3 activity was assessed by flow cytometry.
E, SH-EP cells were treated for 24h with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL in the presence or absence of 50 µM zVAD.fmk. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei and percentage of apoptosis is shown.

In D and E, data represent mean ± SEM of 3 independent experiments performed in triplicate (*p<0.05).

**Figure 3. Activation of Bid and Bax/Bak contributes to Bortezomib-mediated sensitization to TRAIL.**

A, SH-EP cells were treated for 24h with 20 ng/ml TRAIL. Then TRAIL was removed and cells were incubated with 25 µM zVAD in the presence or absence of 50 nM Bortezomib for indicated times. Protein expression of tBid was assessed by Western blot analysis and β-actin was used as loading control (left panel). tBid protein expression was quantified by densitometry (Image J) and percentage of tBid/β-actin level relative to tBid/β-actin level after 24h TRAIL-treatment (0h) is shown (right panel). Data represent mean ± SD of 2 independent experiments.

B, SH-EP cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL for 3h. Protein expression and localization of cytochrome C (Cyt C), Bid, OxPhos Complex IV (COX IV) and α-tubulin were assessed in mitochondrial or cytosolic extracts by Western blot analysis.

C, SH-EP cells were transfected with siRNA against Bid (black bars) or with control siRNA (white bars). Knockdown of Bid protein expression was assessed by Western blotting; β-actin was used as loading control (upper panel). 72h after transfection cells were re-seeded and 24h after re-seeding cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL for indicated times. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei.
D, SH-EP cells were treated either with 50 nM Bortezomib (grey symbols) or 1.25 ng/ml TRAIL (white symbols) alone or in combination (black symbols) at indicated times. Loss of mitochondrial potential (MMP) was assessed by flow cytometry using the fluorescent dye CMXRos (left panel). Cytochrome C release was assessed by flow cytometry using anti-cytochrome C antibody (right panel).

E, SH-EP cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL at indicated times. Bax or Bak activation was analyzed by immunoprecipitation of protein lysates using active conformation-specific anti-Bax or anti-Bak antibodies.

F, SH-EP cells were transfected with siRNA against Bax and Bak (black bars) or with control siRNA (white bars). Knockdown of Bax and Bak protein expression was assessed by Western blotting and β-actin was used as loading control (upper panel).

72h after transfection cells were re-seeded and 24h after re-seeding cells were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL for 8h. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei (lower panel).

In C, D and F, data represent mean + SEM of 3 independent experiments performed in triplicate (*p<0.05, **p<0.0001) (lower panel).

**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 panel). Transduced cells were treated with 50 nM Bortezomib (B) and/or 1.25 ng/ml TRAIL (T) for indicated times; treatment with camptothecin for 24h served as positive control. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei (right panel).
B, SH-EP cells transduced with a vector containing Noxa or control shRNA were treated either with 50 nM Bortezomib (B) and 1.25 ng/ml TRAIL (T) alone or in combination for 3h (left panel) or in combination at indicated times (right panel). Bax or Bak activation was analyzed by immunoprecipitation of protein lysates using active conformation-specific anti-Bax or anti-Bak antibodies. Expression of immunoprecipitated Bak was quantified by densitometry (Image J) and the percentage of Bak/β-actin level is shown.

C, SH-EP cells were transduced with a vector containing p53 or control shRNA. Knockdown of p53 expression was controlled by Western blotting; β-actin served as loading control (left panel). Transduced cells were treated with 50 nM Bortezomib (B) and/or 1.25 ng/ml TRAIL (T) at indicated times; treatment with camptothecin for 24h served as positive control. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei (right panel).

D, SH-EP cells transduced with a vector containing p53 or control shRNA were treated either with 50 nM Bortezomib (B) and 1.25 ng/ml TRAIL (T) alone or in combination for 3h (left panel) or in combination at indicated times (right panel). Bax or Bak activation was analyzed by immunoprecipitation of protein lysates using active conformation-specific anti-Bax or anti-Bak antibodies. Expression of immunoprecipitated Bak was quantified by densitometry (Image J) and the percentage of Bak/β-actin level is shown.

In A and C, data represent mean + SEM of at least three independent experiments performed in triplicate (*p<0.05, **p<0.0001) (right).
Figure 5. Overexpression of Bcl-2 reduces synergistic induction of apoptosis by Bortezomib and TRAIL.

A, SH-EP cells were transduced with mouse Bcl-2 (Bcl-2) or pMSCV control vector (control) and overexpression of Bcl-2 was assessed by Western blotting (left panel). Cells were treated with 50 nM Bortezomib (B) and/or 1.25 ng/ml TRAIL (T) for indicated times (right panel); treatment with camptothecin for 24h served as positive control. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei.

B, Cells were treated either with 50 nM Bortezomib or 1.25 ng/ml TRAIL alone or in combination for 6h. Colonies were stained with crystal violet after 14 days and were counted under the microscope. The percentage of colony numbers compared to the untreated control i (right panel) are shown.

C and D, Cell were treated with 50 nM Bortezomib and/or 1.25 ng/ml TRAIL for 3h (C) or indicated times (D). Protein expression and localization of cytochrome C (Cyt C), Bid, OxPhos Complex IV (COX IV) and α-tubulin were assessed by Western blot analysis (C). Mitochondrial transmembrane potential was assessed by flow cytometry using the fluorescent dye CMXRos (D).

In A and D, data represent mean ± SEM of at least three independent experiments performed in triplicate (*p<0.05, **p<0.0001).

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 24h with 50 nM Bortezomib and/or 100 ng/ml TRAIL. Apoptosis was determined by FACS analysis of DNA fragmentation of propidium iodide stained nuclei and percentage of apoptosis is
shown. Data represent mean + SEM of at least two independent experiments performed in triplicate.

B, Primary cultured neuroblastoma cells (NB16) were treated with 50 nM Bortezomib (B) and/or 100 ng/ml TRAIL (T) for 24h. Expression of caspase-3 was assessed by Western blot analysis. β-actin was used as loading control (right panel). 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 µM Bortezomib alone or in combination daily for two days. Tumor growth was analyzed using hematoxylin/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 death-inducing signaling complex (DISC). See text for more details.
Fig. 1

A

SH-EP

B

Lan-5

SK-N-AS

viability (%) vs. TRAIL (ng/ml)

apoptosis (%) vs. TRAIL (ng/ml)
**Fig. 2**

**A**

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**Counts**

**Fluorescence intensity**
Fig. 3

A

![Bid and β-actin protein levels](image1)

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C

![siRNA control and Bid](image2)

D

![MMP and cytochrome C release](image3)
Fig. 4

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Fig. 5

A

control  Bcl-2

Bcl-2

α-tubulin

B

control  Bcl-2

% specific apoptosis

T  B  BT  T  B  BT  T  B  BT  T  B  BT  CPT

6h  12h  24h  48h  24h

Bortezomib  TRAIL

-  -  +  +  +

mitochondria  cytosol

C

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Cyt C  Bid  tBid  α-tubulin  COX IV

D

control  Bcl-2

loss of MMP (%)

T  B  BT  T  B  BT  T  B  BT

3h  6h  12h

*  **
Fig. 6

A

TRAIL
Bortezomib
Bortezomib + TRAIL

% specific apoptosis

NB1  NB4  NB9  NB10  NB12  NB16

B

NB1

C

B

T

BT

caspase-3

GAP-DH

C

D

Bortezomib

upregulation
stabilization
alteration of pro-
and anti-apoptotic
proteins

TRAIL-R2
p53 Noxa

TRAIL-R2
stabilization

tBid

alteration of pro-
and anti-apoptotic
proteins

FADD
Caspase 8

Bcl2
Bim
Puma

FLIPS
Bid
Bax/Bak

cytochrome C
active
Caspase 9

DISC

Mcl-1

mitochondrion
active
Caspase 8

Mitochondrion
active
Caspase 3

Bortezomib

Mitochondrion
active
Caspase 3

Bortezomib

Mitochondrion
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Caspase 8

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Caspase 3

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Bortezomib primes neuroblastoma cells for TRAIL-induced apoptosis by linking the death receptor to the mitochondrial pathway

Ivonne Naumann, Roland Kappler, Dietrich von Schweinitz, et al.

*Clin Cancer Res* Published OnlineFirst April 1, 2011.

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