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Proteasome Inhibition to Maximize the Apoptotic Potential of Cytokine Therapy for Murine Neuroblastoma Tumors

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Human neuroblastomas possess several mechanisms of self-defense that may confer an ability to resist apoptosis and contribute to the observed difficulty in treating these tumors in the clinical setting. These molecular alterations may include defects in proapoptotic genes as well as the overexpression of prosurvival factors, such as Akt among others. As a key regulator of the turnover of proteins that modulate the cell cycle and mechanisms of apoptosis, the proteasome could serve as an important target for the treatment of neuroblastoma. The present studies provide the first evidence that bortezomib, a newly approved inhibitor of proteasome function, inhibits phosphorylation of Akt, induces the translocation of proapoptotic Bid, and potently enhances the apoptosis of murine neuroblastoma tumor cells in vitro. Furthermore, in that inhibitors of the Akt pathway can sensitize otherwise resistant TBJ/Neuro-2a cells to apoptosis induced by IFN-γ plus TNF-α, we hypothesized that bortezomib also could sensitize these cells to IFN-γ plus TNF-α. We demonstrate for the first time that bortezomib not only up-regulates the expression of receptors for IFN-γ and TNF-α on both TBJ neuroblastoma and EOMA endothelial cell lines, but also markedly enhances the sensitivity of these cells to apoptosis induced by IFN-γ plus TNF-α in vitro. Furthermore, bortezomib enhances the in vivo antitumor efficacy of IFN-γ/TNF-α-inducing cytokines, including both IL-2 and IL-12 in mice bearing well-established primary and/or metastatic TBJ neuroblastoma tumors. Collectively, these studies suggest that bortezomib could be used therapeutically to enhance the proapoptotic and overall antitumor activity of systemic cytokine therapy in children with advanced neuroblastoma. The Journal of Immunology, 2006, 176: 6302–6312.

Neuroblastoma is the most common extracranial solid tumor in children, and the prognosis of patients with high-risk neuroblastoma remains relatively poor overall despite advances in existing therapeutic modalities that include surgery, radiotherapy, and dose-intensive chemotherapy. The emergence of resistance to conventional cytotoxic agents appears to compromise the treatment and long-term prognosis of many neuroblastoma patients. Notably, neuroblastoma tumors may possess molecular alterations that include deficiency and/or silencing of proapoptotic factors such as caspase-3 (1) and caspase-8 (1, 2), loss of the expression of death receptors, including Fas (3) or TRAIL-R2 (4), and overexpression of anti-apoptotic prosurvival factors such as Bcl-2 (5), survivin (6), FLIP (5), and Akt (7). Collectively, these mechanisms may contribute to the observed laboratory and/or clinical resistance of neuroblastoma tumors to existing therapeutic modalities. These observations suggest that improved understanding of the mechanisms that regulate the death of neuroblastoma tumor cells could facilitate the development of novel approaches for the treatment of patients with high-risk neuroblastoma.

Protein degradation via the ubiquitin-proteasome pathway may play a critical role in the regulation of cell cycle progression and apoptosis in both normal and malignant cell populations (8–11). More recently, the U. S. Food and Drug Administration (FDA) has approved bortezomib (velcade, PS-341), a specific reversible inhibitor of the 26S proteasome subunit, for the treatment of patients with multiple myeloma (12). Bortezomib induces apoptosis in several human tumor cell types in vitro (8, 11, 13, 14) and possesses potent antitumor efficacy in preclinical models of prostate (15) as well as breast and lung (16) carcinoma among others. Mechanistically, bortezomib can enhance the expression of key cell cycle and proapoptotic molecules, including p53 (13, 17, 18), p27 (8, 11, 17), p21 (8, 11, 17), Fas/Fas ligand (FasL)3 (19), and TRAIL-R2 (19). Furthermore, bortezomib can block the activation of NF-κB (17, 19–22) and abrogates the expression/activity of prosurvival factors, such as c-FLIP (19), Bcl-xL (8), Bcl-2 (8, 19, 22), c-IAP-2 (19), and Akt (23, 24). Collectively, these observations suggest that proteasome inhibitors such as bortezomib could overcome molecular mechanisms that contribute to the resistance of neuroblastoma tumors to apoptosis.

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3 Abbreviations used in this paper: FasL, Fas ligand; RFP, red fluorescent protein; TMEM, tetramethylmethanamine methyl ester; i.s., intrasplenic; tBid, truncated Bid; IκB, IκB; IRF-1, IFN regulatory factor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt.
The present studies were designed to investigate whether bortezomib could induce apoptosis of neuroblastoma tumor cells and mediate the regression of established neuroblastoma tumors in vivo. We found that bortezomib induces apoptosis of TBJ neuroblastoma tumor cells in vitro and do so in conjunction with inhibition of Akt phosphorylation and induction of the translocation of Bid, an important mediator of mitochondria-dependent pathways of apoptosis (25). Furthermore, bortezomib up-regulates TNF-α and IFN-γ receptor expression on TBJ cells and sensitizes these cells to apoptosis induced by TNF-α plus IFN-γ. We subsequently found that systemic administration of bortezomib can enhance the antitumor activity of IFN-γ/TNF-α-inducing cytokines, including IL-2 and IL-12, in preclinical models of primary and/or metastatic murine neuroblastoma. Our observations suggest that the inhibition of proteasome function in tumor cells could be highly effective in reducing the threshold for apoptosis induction by IFN-γ/TNF-α-inducing cytokines and provide preclinical rationale for a novel therapeutic use of proteasome inhibitors to potentiate the proapoptotic effects and antitumor efficacy of cytokines.

Materials and Methods

Animals and cell lines

Murine Neuro-2a neuroblastoma and EOMA hemangiendothelioma tumor cell lines were purchased from the American Type Culture Collection. TBJ is a metastatic subclone of Neuro-2a and was provided by Dr. M. Ziegler (Children’s Hospital, Boston, MA). TBJ and Neuro-2a neuroblastoma cells, syngeneic to AJ mice, were maintained via serial passage in vivo. TBJ cells transfected to overexpress the red fluorescent protein (TBJ-RFP) were generated as described previously (26). Where indicated, TBJ cells transfected with a commercially available mammalian expression vector pEDEGFP-BID (BD Biosciences) were used. This vector encodes destabilized enhanced GFP fused to the C terminus of human Bid protein and contains a neomycin resistance gene allowing for selection of TBJ cells that have been stably transfected to overexpress this vector (TBJ-Bid-GFP). Where indicated, TBJ cells transfected with pRK-GFP plasmid (provided by Dr. K. Yamada, National Institutes of Health, Bethesda, MD) along with commercially available pSUPER.retro.puro vector (Oligoengine) were used (TBJ-GFP). These cells overexpress GFP alone as well as the puroycin resistance gene as a selectable marker. Transfection of cells with the pEDEGFP-BID, pRK-GFP, and pSUPER.retro.puro plasmids, respectively, was performed using the standard FuGENE method (Roche). Expression of unmodified GFP and EGFP-Bid was confirmed by fluorescence microscopy, and expression of EGFP-Bid also was confirmed as described below by Western blot analysis of tumor cell lysates. The newly established POB UN303 murine neuroblastoma cell line was derived from a spontaneous neuroblastoma tumor arising in an N-Myc transgenic mouse, and has been described elsewhere in detail (J. K. Stauffer et al., manuscript in preparation). For in vitro studies, all cell lines were maintained in M1 medium 1640 containing essential amino acids, penicillin, streptomycin, and 10% FCS at 37°C in a 5% CO2-containing humidified chamber. Male A/J mice were purchased from the Animal Production Area (Charles River, Frederick, MD) and were generally used at 8–10 wk of age. Animal care was provided in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86-23, 1985).

Reagents

Commercially available bortezomib (Millennium Pharmaceuticals) was reconstituted according to the manufacturer’s instructions and diluted in 0.9% normal saline before in vivo administration. Commercially available recombinant human IL-2 (Chiron) and murine IL-12 (specific activity ≥1 × 10^6 U/mg; PeproTech) were prepared according to the manufacturer’s instructions before in vivo administration. Recombinant murine TNF-α (spec. act. ≥1×10^8 U/mg) and murine IFN-γ (spec. act. ≥1×10^7 U/mg) were obtained from PeproTech. rhsSuper FasL and rhsTRAIL were purchased from Alexis Biochemicals. The mitochondrial MitoTracker Deep Red 633 dye and tetramethylrhodamine methyl ester (TMRM) perchlorate were purchased from Molecular Probes (Invitrogen Life Technologies). The mitochondrial MitoTracker Deep Red, the mitochondrial MitoTracker Green, and the LumiGene (Luc) and β-galactosidase enzyme assay kits were purchased from Promega. The Luc vector pXP2 containing the 1312 IFN regulatory factor 1 (IRF-1) gene promoter (5′-Δ-1312-Luc construct) was a gift from Dr. H. Young (NCI-Frederick, Frederick, MD) and has been described previously (27).

Antibodies

PE-labeled hamster anti-mouse Fas (Jo-2), purified hamster anti-mouse TNF-R1 (5p5), purified hamster anti-mouse IFN-γ-Rα, purified hamster anti-mouse IFN-γ-Rβ, biotin-conjugated mouse anti-hamster IgG, PE-labeled streptavidin, hamster anti-mouse IgG isotype control, and purified monoclonal mouse anti-mouse/rat SHIP-1 AbS were purchased from BD Pharmingen. The monoclonal rat anti-mouse Bid Ab that also recognizes human Bid was obtained from R&D Systems. The MDS-1 hamster anti-mouse TRAIL-R2 (DR5) Ab was purchased from eBioscience. The polyclonal rabbit anti-mouse PTEN, polyclonal rabbit anti-mouse Akt, and phosphospecific polyclonal rabbit anti-mouse Akt (serine 473) Abs were obtained from Cell Signaling Technology. The monoclonal mouse anti-α-tubulin Ab (clone DM1A) was purchased from Calbiochem. The HRP-conjugated anti-mouse IgG and goat anti-rabbit IgG were purchased from Bio-Rad. Peroxidase-labeled goat anti-rabbit IgG was obtained from Kirkegaard & Perry Laboratories.

In vitro tumor/endothelial studies

Proliferation assay. To investigate the effect of bortezomib on tumor cell proliferation, TBJ Neuro-2a and POB UN303 tumor cells (1 × 10^5 cells/well) were incubated in triplicate for 24, 48, and 72 h in 96-well plates with various concentrations of bortezomib. Where indicated, cells were either pulsed with [3H]thymidine (1 μCi/well) 16 h before harvest to determine [3H]thymidine incorporation or incubated with a combined solution of the tetracyclol acid compounds (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphosphonic acid) (MTT) and the electrically neutral reagent phenamethsulfate to determine the quantity of formazan product formed by the reduction of MTS reagent by viable cells. The absorbance of formazan at 490 nm is directly proportional to the number of viable cells in culture. Similar assays were performed using POB UN303 murine neuroblastoma tumor cells treated for 72 h with various concentrations of bortezomib. The MTS data shown are corrected for background absorbance for each well.

Annexin V/7-MU assay. Preadhered neuroblastoma (Neuro-2a, TBJ, and UN303) cells were cultured with bortezomib for 48 h. Cells were subsequently harvested and stained with either Annexin V-FITC or 100 nM red potentiometric dye TMRM for assay of apoptosis and/or loss of mitochondrial membrane potential respectively using a FACScan flow cytometer and Cell Quest software (BD Biosciences). Where indicated, TBJ neuroblastoma cells were incubated with bortezomib for 4 h and then treated with FasL (100 ng/ml), TRAIL-A2 (200 ng/ml), IFN-γ (100 IU/ml) plus TNF-α (50 ng/ml), or medium alone for a additional 20 h. In follow-up studies, preadhered TBJ or Neuro-2a cells also were incubated with bortezomib (5 or 10 nM) or medium alone for 4 h, followed by treatment with IFN-γ (100 IU/ml) for 20 or 44 h before Annexin V staining as above. Where indicated, EOMA endothelial cells were treated similarly with bortezomib (10, 30, 50, or 100 nM) for 44 h, incubated for 18 h with either Annexin V-FITC or medium only, then incubated with Annexin V-FITC and analyzed by FACScan as above.

Western blotting. Preadhered Neuro-2a, TBJ, or TBJ-Bid-EGFP neuroblastoma cells were treated with bortezomib for 13 or 24 h where indicated, and total protein lysates were extracted from cultured cells using standard techniques. Protein concentrations were determined using the BCA protein assay reagent (Pierce). Equal amounts of protein boiled in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86-23, 1985).  

Confocal microscopy. TBJ-Bid-EGFP tumor cells (2 × 10^5) were grown overnight in 35-mm glass-bottom microwell polyn-l-lysin coated dishes (MatTek Cultureware). Cells were incubated with either bortezomib (50 nM) or fresh medium alone for 13 h. Old medium from cells was then replaced with fresh medium containing MitoTracker Deep Red (500 nM) for mitochondrial staining. Cells were incubated for 45 min at 37°C and subsequently analyzed for subcellular mitochondrial Bid-EGFP localization using a confocal laser-scanning microscope (LSM 510; Zeiss).
A 488-nm Ar laser with a 500- to 550-nm band-pass emission filter and a 633-nm HeNe laser with a 646- to 753-nm band-pass emission filter were used. The objective used was a Zeiss Plan-Apochromat 63X/1.4 NA differential interference contrast oil lens. TBJ-GFP cells treated similarly with bortezomib also were imaged as negative controls to confirm that the altered distribution of GFP-Bid in TBJ-Bid-EGFP cells treated with bortezomib was consistent with cleavage and mitochondrial translocation of Bid.

Modulation of cell surface receptor expression. To investigate the impact of bortezomib on cell surface expression of Fas and TRAIL-R2, TBJ or EOMA cells were incubated with bortezomib (5–30 nM) or medium alone for 24 h. Cells were then harvested and stained with either PE-labeled hamster anti-mouse TRAIL-R2 (MD5–1), PE-labeled hamster anti-mouse Fas Ab (Jo-2), or PE-labeled hamster anti-mouse IgG isotype control Ab. The expression of cell surface TNF-R1 or IFN-γ-R also was evaluated on TBJ and EOMA cells cultured under similar conditions. Cells were labeled with purified hamster anti-mouse TNF-R1 (p55), purified hamster anti-mouse IFN-γ-Rα, purified hamster anti-mouse IFN-γ-Rβ, or hamster anti-mouse IgG isotype control Ab, followed by staining with biotin-conjugated mouse anti-hamster IgG and PE-labeled strepavidin. Cells were fixed in 1% paraformaldehyde in PBS and analyzed using a FACScan flow cytometer and Cell Quest software.

Luc activity assays. Preadhered TBJ cells (2 × 10⁵) were transiently transfected with 1 µg of the human 5'-Δ-1312-Luc construct containing a 1.3-kb-long IRF-1 promoter fragment that extended from −1312 to +7 (27) and 0.5 µg of pSV-β-galactosidase control vector to monitor for transfection efficiency. The next day, cells were treated with bortezomib (10 nM) or fresh medium alone for 24 h, followed by bortezomib (10 nM) ± IFN-γ (100 IU/ml) or fresh medium alone for an additional 24 h. Cells were finally lysed according to the manufacturer’s instructions (Promega), and luminiscence was measured in a luminometer (Lumat LB9501; EG & G Berthold).

Tumor models

Cohorts of 10 A/J mice per group were used in all therapy studies. To establish s.c. primary tumors, mice were injected s.c. in the mid-flank with syngeneic murine TBJ neuroblastoma tumor cells (1.2 × 10⁶ cells/animal in 0.2 ml HBSS). Tumors were allowed to become well established for 6 days after tumor cell injection. To induce disseminated hepatic and/or pulmonary neuroblastoma metastases, mice were injected i.v. with TBJ-RFP cells (1 × 10⁵ cells/animal in 0.2 ml HBSS), and metastatic tumors were allowed to become well established for 5 days after tumor cell injection. Where indicated, mice were injected intrasplenically (i.s.) with TBJ-RFP cells (2.5 × 10⁵ cells/animal in 0.5 ml HBSS), and selective hepatic metastases were allowed to become well established for 5 days after tumor cell injection.

FIGURE 1. Bortezomib inhibits the proliferation/viability of murine TBJ, Neuro-2a, and POB UN303 neuroblastoma cells. TBJ, Neuro-2a, and POB UN303 tumor cells were incubated with varying concentrations of bortezomib for a total of 72 h as described in Materials and Methods. For proliferation assays, cells were pulsed with [³H]thymidine 16 h before harvest. Complementary studies were performed using the MTS assay as described in Materials and Methods. Bortezomib inhibited proliferation of TBJ (A), Neuro-2a (B), and POB UN303 (C) neuroblastoma tumor cells in a dose- and time-dependent manner (D). Similar observations were obtained using the MTS assay for TBJ (A), Neuro-2a (B), and POB UN303 (C). Data are the mean ± SD for triplicate samples. Background values for absorbance in control wells were subtracted for the MTS assay.
Treatment of established s.c. primary tumors. To investigate the antitumor activity of combined administration of bortezomib and IL-2, mice bearing well-established day-6 SC-TBJ tumors were injected i.p. each morning with IL-2 (50,000 IU in 0.2 ml of HBSS containing 0.1% homologous serum) or vehicle alone on days 6–10, 13–17, and 20–24 after tumor implantation. Bortezomib (20 μg in 0.2 ml 0.9% normal saline) or vehicle alone was administered i.p. in the afternoon on days 7, 10, 14, 17, 21, and 24 after tumor implantation. Mice were monitored for tumor growth, and bidirectional tumor dimensions were determined using calipers. Estimated tumor volumes were then determined by calculating the product of the smallest measured tumor dimension squared multiplied by the largest measured tumor dimension.

Treatment of established induced neuroblastoma metastases. To investigate the ability of bortezomib to potentiate the antitumor activity of IL-2 in mice bearing widespread metastases, mice were injected i.v. with TBJ-RFP tumor cells as described above. After 5 days to allow metastatic tumors to become well established, IL-2 (50,000 IU in 0.2 ml of HBSS containing 0.1% homologous serum) or vehicle alone was administered i.p. in the morning on days 5–9 and 12–15 after tumor implantation. Bortezomib was administered i.p. in the afternoon on days 6, 9, and 13 after tumor implantation. Mice were euthanized on day 16 after tumor implantation, and livers were resected individually and stored in cold PBS before fluorescent imaging of metastases as described above. To investigate whether bortezomib could potentiate the antitumor activity of IL-2 in mice bearing induced metastases, mice were injected i.v. with TBJ-RFP tumor cells as described above. After 5 days to allow hepatic metastases to become well established, IL-12 (0.1 μg in 0.2 ml containing 0.1% homologous serum) or vehicle alone was administered i.p. in the morning on days 5, 8, and 12 after tumor implantation. Bortezomib (20 μg in 0.2 ml 0.9% saline) or vehicle alone was delivered i.p. 5–6 h later the same day. Mice were euthanized on day 13 after tumor injection, and livers were resected and placed into cold PBS. The impact of therapy on metastatic disease burden in the liver was then evaluated using fluorescent imaging as outlined below.

Fluorescent imaging. Macroscopic imaging of hepatic TBJ-RFP metastases was performed using a slit fiber optic illuminated light table (Lighttools Research), and images were captured by a zoom lens equipped Nikon DXM1200 digital camera. A Nikon SMZ800 stereomicroscope equipped with a mercury lamp and a Nikon DXM1200 digital camera was used to collect low-power (×10–63 magnification) images. RFP fluorescence was induced by excitation at 540 nm and collected through a 590 nm filter.

Statistical methods. The Jonckheere-Terpstra test for trend was used to compare tumor volumes among the respective control, bortezomib, IL-2, or bortezomib/IL-2 treatment groups in mice bearing s.c. TBJ tumors. All p values were considered significant at p \( \leq 0.05 \).

Results

Bortezomib inhibits proliferation and induces apoptosis of murine neuroblastoma cell lines

We have shown previously that TBJ and Neuro-2a murine neuroblastoma tumors are intrinsically resistant to receptor-mediated apoptosis (28). More specifically, these cells demonstrate limited expression of death receptors, including Fas and TRAIL-R2, and express high levels of phosphorylated Akt, a key anti-apoptotic factor (28). In that prosesosome inhibitors can both up-regulate the expression of proapoptotic genes (8, 11, 17–19), and down-regulate the expression/activity of prosurvival factors, such as Akt in hematologic neoplasms (23, 24), we hypothesized that bortezomib could mediate antitumor effects against neuroblastoma tumors as well. TBJ, Neuro-2a (A/J background), and the N-Myc overexpressing POB UN303 (C57BL/6J background) murine neuroblastoma cells were treated with bortezomib, and the proliferative capacity of tumor cells was determined by incorporation of [\(^{3}H\)]thymidine or the MTS assay as described in Materials and Methods. At day 3 after treatment, incorporation of [\(^{3}H\)]thymidine in TBJ, Neuro-2a, and POB UN303 cells was inhibited by 70, 65, and 96%, respectively, compared with controls at 20-nM concentrations of bortezomib (Fig. 1, A–C). Similar results were noted when using the MTS assay (Fig. 1, A–C). At day 3 after treatment, bortezomib (20 nM) inhibited the proliferation/viability of TBJ, Neuro-2a, and POB UN303 cells by 62, 50, and 91%, respectively, compared with controls as assessed by MTS assay. Furthermore, the inhibitory effects of bortezomib on neuroblastoma cell proliferation were not only dose dependent as shown above, but also time dependent (Fig. 1D). In this regard, similar results were seen based on [\(^{3}H\)]thymidine incorporation (Fig. 1D) as well as MTS assay (data not shown), with time-dependent inhibition of proliferative responses by both TBJ and Neuro-2a cells at concentrations of bortezomib >10 nM. Bortezomib treatment also induced apoptosis of murine neuroblastoma cells as assessed by Annexin V staining and decreased mitochondrial membrane potential as assessed by TMRM staining (Fig. A–C). TMRM accumulates in polarized mitochondria but is released after mitochondrial depolarization resulting in loss of TMRM signal in apoptotic cells. Among TBJ cells treated with bortezomib, only 11 ± 1% of cells stained positive for TMRM after 2 days of exposure to bortezomib (20 nM), compared with 91 ± 1% of control cells treated with medium alone (Fig. 2A). Similarly, among Neuro-2a cells, only 26 ± 0.6% of cells were TMRM positive after 2 days of exposure to bortezomib (20 nM), compared with 86 ± 2% of control cells treated with medium alone (Fig. 2B). Using POB UN303 cells, only 43 ± 0.8% of cells stained positive for TMRM after 2 days of exposure to bortezomib (20 nM), compared with 82 ± 0.03% of
Bortezomib inhibits Akt phosphorylation and induces Bid translocation in murine neuroblastoma cells

Bortezomib was able to induce marked apoptosis in both TBJ and Neuro-2a cells that express high levels of phosphorylated Akt (active) and are intrinsically resistant to apoptosis. Consequently, we investigated whether bortezomib could inhibit the activity of Akt and/or induce the expression/activity of proapoptotic factors in these cells. TBJ and Neuro-2a tumor cells were treated with bortezomib or vehicle control for 24 h, and total cellular lysates were analyzed by Western blotting for Akt expression/phosphorylation. Even at low nanomolar concentrations, bortezomib markedly inhibited the phosphorylation of Akt (Ser-473) in both TBJ and Neuro-2a tumor cells, while the levels of total Akt remained essentially unaltered (Fig. 3A). Because phosphorylation of Akt on Ser-473 is required for maximum Akt activity (29, 30), our data suggested that bortezomib inhibited Akt function in both TBJ and Neuro-2a neuroblastoma cells. We also investigated whether inhibition of Akt phosphorylation by bortezomib was due to inhibition of the degradation of AKT phosphatases. Bortezomib does not appear to induce decreases in Akt phosphorylation (Ser-473) by altering the expression of PTEN and SHIP-1 phosphatases in murine neuroblastoma cells (data not shown).

Next, we investigated whether inhibition of AKT phosphorylation/activation by bortezomib resulted in enhanced activity of Bid, a proapoptotic Bcl-2 family member that triggers mitochondrial depolarization but is normally suppressed by activated Akt (31, 32). During immune/receptor-mediated apoptosis, cytoplasmic Bid is proteolytically cleaved (activated) to form truncated Bid (tBid). tBid then translocates to the mitochondria (25), where it contributes to mitochondria-dependent pathways for apoptosis. Treatment with bortezomib induced apoptosis of murine neuroblastoma cells in conjunction with marked reductions in mitochondrial membrane potential. To directly investigate whether bortezomib also modulated the activation and subcellular translocation of Bid, TBJ cells were engineered to overexpress a commercially available fusion construct consisting of GFP fused to the C terminus of human Bid as described previously (TBJ-Bid-EGFP) (28). TBJ cells that overexpress unmodified GFP alone without Bid (TBJ-GFP) also were generated as an additional negative control cell. TBJ-Bid-EGFP and TBJ-GFP cells were treated with bortezomib (50 nM) or medium alone for 13 h. In TBJ-Bid-EGFP cells treated with medium alone, a diffuse pattern of GFP expression is noted consistent with cytosol of TBJ cells (B and D, respectively). In cells treated with bortezomib, Bid-EGFP is translocated to the mitochondria, as evidenced by a punctate pattern of green fluorescence that colocalizes with red mitochondria (C, see arrows). In contrast, in TBJ cells expressing unmodified GFP alone, a diffuse cytoplasmic pattern of green fluorescence is still observed after treatment with bortezomib (E, see arrows). Equal amounts of protein lysates from TBJ-Bid-EGFP cells treated with bortezomib for 13 h also were analyzed by Western blot for determination of Bid-EGFP cleavage (F). Equal loading of protein samples was confirmed by stripping and reprobing the Bid-EGFP blot for α-tubulin. Blots for pAkt (Ser-473) and total Akt were from separate gels using the same cell lysates.
...translocation to areas of mitochondrial aggregation (as assessed via MitoTracker Deep Red 663 staining) resulting in a striking punctuate pattern of GFP distribution that is consistent with cleavage (activation) and translocation of Bid from the cytosol to the mitochondria (Fig. 3C). This distribution of Bid-EGFP was not due to nonspecific aggregation of Bid-EGFP protein in cells undergoing apoptosis as TBJ cells expressing GFP alone (TBJ-GFP) did not exhibit a similar punctate pattern of GFP distribution after bortezomib treatment (Fig. 3, D and E). Bortezomib induced cleavage of full-length Bid-EGFP as early as 13 h and at concentrations as low as 20 nM, and cleavage of Bid-EGFP correlated closely with observed decreases in Akt phosphorylation (Ser-473) in TBJ-Bid-EGFP cells (Fig. 3F). Similar findings were observed at 24 h of treatment with bortezomib (data not shown). Cleavage of endogenous Bid also was observed in both TBJ and TBJ-Bid-EGFP cells cultured under similar conditions (data not shown).

**Bortezomib up-regulates IFN-γ and TNF-α receptor expression and sensitizes TBJ neuroblastoma cells to apoptosis induced by IFN-γ plus TNF-α**

We have shown previously that, although TBJ neuroblastoma cells express receptors for TNF-α, they express no Fas and only negligible amounts of TRAIL-R2 (28). Treatment of TBJ cells with bortezomib (5 nM) for 24 h did not result in any changes in the cell surface expression of either Fas or TRAIL-R2, but did increase the expression of TNF-R1 (control = 11 ± 5% vs bortezomib = 44 ± 20%) (Fig. 4A). In that IFN-γ can enhance the sensitivity of several malignant cell types to TNF-α in vitro (33, 34), we also investigated whether bortezomib could modulate the expression of the IFN-γ-R on TBJ cells. The IFN-γ-R is composed of a ligand binding subunit (IFN-γ-Rα) and a signaling subunit (IFN-γ-Rβ) (35, 36). TBJ cells express low levels of IFN-γ-Rβ, and expression of this subunit is not up-regulated by treatment with bortezomib. In contrast, bortezomib markedly enhances cell surface expression of IFN-γ-Rα subunit on TBJ cells (control = 13 ± 0.8% vs bortezomib at 5 nM = 24 ± 7% vs bortezomib at 10 nM = 53 ± 7%) (Fig. 4B). To determine whether an increase in IFN-γ-Rα subunit is sufficient to increase cellular responses to IFN-γ, TBJ cells were cotransfected with the 5′Δ-1312-Luc construct containing the human IRF-1 promoter fragment to monitor for IFN-γ responsiveness along with the pSV-β-galactosidase control vector to monitor for transfection efficiency. IRF-1 is an IFN-γ-inducible transcriptional factor that can mediate secondary IFN-γ responses, including up-regulation of MHC class I and II and expression of the chemokine IFN-inducible protein 10 (37).Transiently transfected TBJ cells that were first pretreated with bortezomib (10 nM) for 24 h and then stimulated with IFN-γ (100 IU/ml) showed small but consistent increases in IRF-1 promoter activity (75 ± 17 × 10³ U), compared with those that were treated with bortezomib (3 ± 0.2 × 10³ U), IFN-γ (42 ± 8 × 10³ U), or medium alone (2 ± 0.5 × 10³ U) (Fig. 4C). These data confirmed that up-regulation of IFN-γ-Rα by bortezomib also increases the responsiveness of TBJ tumor cells to IFN-γ.

We have shown previously that inhibitors of protein synthesis (cycloheximide) or small molecule inhibitors of PI3K/Akt (LY294002/SH5) also can sensitize TBJ or Neuro-2a cells to apoptosis induced by IFN-γ/TNF-α in vitro (28). In that bortezomib both inhibits Akt activity in murine neuroblastoma cells and enhances cell surface expression of receptors for TNF-α and IFN-γ, we hypothesized that bortezomib could sensitize TBJ or Neuro-2a tumor cells to apoptosis induced by TNF-α ± IFN-γ. Cells were pretreated with bortezomib (10 or 20 nM) for 4 h and then incubated with FasL (100 ng/ml), TRAIL (200 ng/ml), IFN-γ (100 IU/ml) plus TNF-α (50 ng/ml), or medium alone for an additional

**FIGURE 4.** Bortezomib enhances the expression of TNF-R1 and IFN-γ-Rα in TBJ neuroblastoma cells. Tumor cells were treated with bortezomib for 24 h and then stained with Abs directed against Fas, TRAIL-R2, TNF-R1, or IFN-γ-Rα, or IFN-γ-Rβ or the appropriate isotype-matched control Ab and were analyzed by flow cytometry as described in Materials and Methods. Bortezomib increases the expression of TNF-R1 and IFN-γ-Rα but not of Fas, TRAIL-R2, or IFN-γ-Rβ on TBJ cells (A and B). For the histograms (A and B), the black dotted line represents isotype IgG control for TBJ cells treated with medium alone; the red dotted line represents isotype IgG control for TBJ cells treated with bortezomib; the black solid line represents staining for Fas, TRAIL-R2, TNF-R1, IFN-γ-Rα, or IFN-γ-Rβ for TBJ cells treated with medium alone; and the red solid line represents staining for Fas, TRAIL-R2, TNF-R1, IFN-γ-Rα, or IFN-γ-Rβ for TBJ cells treated with bortezomib. For the bar graphs (A and B), data shown represents experimental values for each group after subtraction of isotype control values. Results are representative of at least two separate experiments. Data are the mean ± SE for duplicate samples.
Bortezomib increased the sensitivity of TBJ cells to IFN-γ plus TNF-α but not to FasL or TRAIL-mediated apoptosis (as assessed via Annexin V-positive cells) (Fig. 5A). Thus, at day 1 after treatment with bortezomib (20 nM), followed by IFN-γ plus TNF-α, 72 ± 0.2% of TBJ cells were Annexin V positive, compared with 24 ± 2% of cells treated with bortezomib followed by FasL, 23 ± 1% of cells treated with bortezomib followed by TRAIL, 27 ± 1% of cells treated with bortezomib followed by medium alone, and only 2 ± 0.3% of cells incubated with medium alone at both steps. To define the relative roles of TNF-α or IFN-γ alone in cells sensitized to undergo apoptosis when treated with bortezomib, followed by IFN-γ plus TNF-α, TBJ cells were pretreated with bortezomib (5 or 10 nM) as outlined above and then incubated with IFN-γ (100 IU/ml) ± TNF-α (50 ng/ml) for an additional 44 h. Although TNF-α is the dominant contributor to the induction of apoptosis in TBJ cells treated with bortezomib, followed by IFN-γ plus TNF-α, additive enhancement is achieved when cells are treated with the combination of these two cytokines (Fig. 5B). Thus, 26 ± 2% of TBJ cells treated with bortezomib (10 nM) followed by TNF-α alone were Annexin V-positive, compared with 6 ± 0.2% of TBJ cells treated with bortezomib followed by IFN-γ alone, 33 ± 3% of cells treated with bortezomib followed by the combination of IFN-γ plus TNF-α, 4 ± 0.3% of cells treated with bortezomib followed by medium alone, and 1 ± 0.5% of cells treated with medium alone at both steps. Similar trends in the induction of apoptosis were observed in Neuro-2a cells after treatment with bortezomib and TNF-α ± IFN-γ (data not shown).

To investigate whether bortezomib also could also modulate apoptosis in endothelial cell populations, we used EOMA, a murine microvascular endothelial cell line. Pre-exposure of EOMA cells to bortezomib markedly enhanced their subsequent sensitivity to IFN-γ plus TNF-α-mediated apoptosis (Fig. 5C). At day 1 after treatment with IFN-γ plus TNF-α and bortezomib (10 nM), 81 ± 0.4% of EOMA cells were apoptotic as evidenced by Annexin V positivity, compared with 10 ± 0.2% of cells treated with bortezomib alone, 34 ± 0.1% of cells treated with IFN-γ plus TNF-α alone, and 7 ± 0.02% of control cells treated with medium alone. Bortezomib also enhanced cell surface expression of IFN-γ-Rα on EOMA cells in vitro (Fig. 5D), and although bortezomib did not induce large increases in cell surface TNF-R1 expression by EOMA cells, ~50% of EOMA cells did express TNF-R1 constitutively (data not shown). Our results suggest that bortezomib can enhance IFN-γ and TNF-α receptor expression on tumor and/or endothelial cells in vitro and sensitize these cells to apoptosis induced by IFN-γ plus TNF-α.

**FIGURE 5.** Bortezomib enhances sensitivity of TBJ neuroblastoma and endothelial EOMA cells to apoptosis induced by treatment with IFN-γ plus TNF-α. A, TBJ cells were incubated with bortezomib (10 or 20 nM) for 4 h before treatment with either FasL (100 ng/ml) or TRAIL (200 ng/ml) or IFN-γ (100 IU/ml) plus TNF-α (50 ng/ml). Cells were harvested at 24 h, stained with Annexin V-FITC, and analyzed by flow cytometry as described in Materials and Methods. B, In follow-up studies, TBJ cells were treated with bortezomib (5 and 10 nM), followed by IFN-γ (100 IU/ml) ± TNF-α (50 ng/ml) to assess the relative contribution of these cytokines. Cells were harvested at 48 h and analyzed by flow cytometry as described above. Data are the mean ± SE of duplicate samples. C, EOMA murine endothelial cells also were treated similarly with bortezomib followed by IFN-γ (100 IU/ml) plus TNF-α (50 ng/ml) as described in Materials and Methods. After 24 h, cells were stained with Annexin V-FITC and analyzed by flow cytometry as above. D, Bortezomib also increases the expression of IFN-γ-Rα on EOMA cells. Cells were exposed to bortezomib for 24 h, and expression of IFN-γ-R was determined by flow cytometry as above. For the histograms, the black dotted line represents isotype IgG control for TBJ cells treated with medium; the black solid line represents IFN-γ-Rα or IFN-γ-Rβ for TBJ cells treated with medium; the red dotted line represents isotype IgG control for TBJ cells treated with bortezomib; and the red solid line represents FN-γ-Rα or IFN-γ-Rβ for TBJ cells treated with bortezomib (D). For the bar graph (D), data shown represent experimental values for each group after subtraction of isotype control values. Data are the mean ± SE for duplicate samples.
Bortezomib potentiates the antitumor activity of IL-2 in mice bearing well-established primary TBJ neuroblastoma tumors

We hypothesized that bortezomib could potentiate the proapoptotic and overall antitumor activity of potent IFN-γ/TNF-α-inducing cytokines such as IL-2 or IL-12 in vivo. Cohorts of mice bearing well-established SC-TBJ tumors were treated with IL-2 ± bortezomib or vehicles alone as described in Materials and Methods. Combined administration of IL-2 and bortezomib delayed progression of SC-TBJ tumors more effectively than either of the single agents alone (Fig. 6). A highly significant reduction in tumor size was observed as early as day 9 post tumor implantation (day 3 of therapy) (p = 0.009) and was sustained through day 27 post tumor implantation (day 21 of therapy) (p = 0.001).

**Bortezomib potentiates the antitumor activity of IL-2 and IL-12 in mice bearing established metastatic neuroblastoma**

To investigate whether bortezomib could potentiate the antitumor activity of cytokine therapy in a more therapeutically-challenging setting of induced metastatic disease, mice bearing day 5 i.v. induced TBJ-RFP metastases were treated with bortezomib ± IL-2 or vehicles alone as described in Materials and Methods and were then euthanized on day 11 of therapy (day 16 after tumor injection). Individual livers were then resected, and the metastatic disease burden in each liver was imaged via conventional light and fluorescence microscopy as described in Materials and Methods. Systemic administration of bortezomib in combination with IL-2 inhibited the growth of induced hepatic TBJ-RFP metastases more effectively than either single agent alone (Fig. 7A). This observation also is demonstrated in higher-magnification views of representative individual organs using both conventional light (Fig. 7B) and fluorescent (Fig. 7C) imaging. Furthermore, as shown in light microscopy as described in Materials and Methods, systemic administration of bortezomib plus IL-2 had the largest reduction in tumor size as well as grossly apparent decreases in tumor vascularity (D).

**DAYS POST TUMOR IMPLANTATION**

**FIGURE 6.** Combined administration of IL-2 and bortezomib delays primary SQ-TBJ neuroblastoma tumor growth more effectively than either of the single agents alone. Mice bearing well-established SQ-TBJ tumors were established as described in Materials and Methods. Mice were treated with either IL-2 (50,000 IU) or vehicle alone i.p. on days 6–10, 13–17, and 20–24 after tumor implantation. Bortezomib (0.8 mg/kg) or vehicle alone was delivered i.p. on days 7, 10, 14, 17, and 21 after tumor implantation. Administration of bortezomib in combination with IL-2 inhibited TBJ tumor growth more effectively over time (days 9–27 after tumor implantation) than either of the single agents alone (p = 0.05 for bortezomib plus IL-2 vs bortezomib alone or vs IL-2 alone or vs vehicle control).
effects associated with administration of high-dose cytokines can be substantial. Furthermore, many tumors initiate a range of mechanisms that can actively subvert the host antitumor immune response. These observations suggest that successful approaches for the immunotherapy of solid tumors will most likely evolve from rationally designed combinations of agents with complementary mechanisms of action. The present studies describe a novel approach using bortezomib, a recently described inhibitor of proteasome function, that not only counters mechanisms of tumor self-defense, but also mediates dramatic antitumor effects against murine neuroblastoma tumors when used in combination with potent immunoregulatory cytokines.

When administered alone or in combination, IFN-γ/TNF-α-inducing cytokines, including IL-2 (44), IL-12 (45, 46), and IL-18 (47) mediate potent therapeutic effects in several preclinical tumor models. The antitumor mechanisms engaged by IL-12 or IL-18 are critically dependent on the induction of endogenous IFN-γ production, and more specifically, the ability of tumor cells to respond to IFN-γ (47–50). In turn, IFN-γ can directly sensitize tumor and/or endothelial cell populations to receptor-mediated apoptosis induced by Fas/FasL (3, 33, 51), TRAIL/TRAIL-R (4, 33, 34), or TNF/TNF-R (33, 34) in vitro, and the antitumor activity of IL-12 or IL-18 administered alone or in combination with IL-2 appears to be mediated by the Fas/FasL (50, 52) and/or TRAIL/TRAIL-R (53) pathways in vivo. Similarly, a positive correlation exists between clinical response to IL-2 therapy and the sustained production of endogenous TNF-α (54). Thus, downstream cytokines, including IFN-γ and TNF-α, may be particularly important mediators of the antitumor activity of cytokines such as IL-2, IL-12, and IL-18.

The molecular mechanisms that mediate the response of neuroblastoma tumors to immunotherapy appear to be considerably more complex. Human neuroblastoma tumors possess a range of molecular alterations that may contribute to an intrinsic resistance to receptor-induced apoptosis, including defects in the expression and/or function of proapoptotic genes, including caspases (1, 2), Fas (3), and TRAIL-R2 (4) as well as overexpression of a range of prosurvival factors (5–7), most notably Akt (7). We have shown previously that murine neuroblastoma tumors (TBJ/Neuro-2a) also express high levels of activated phosphorylated Akt, and that inhibitors of Akt (SH5) can sensitize these otherwise resistant cells to apoptosis induced by IFN-γ plus TNF-α (28). Furthermore, systemic administration of IL-12 can inhibit Akt phosphorylation and up-regulate proapoptotic genes within established TBJ tumors in vivo in conjunction with marked enhancement of both tumor and endothelial cell apoptosis and the induction of tumor regression in treated mice (28). Collectively, these observations suggest that targeting mechanisms that regulate tumor self-defense (e.g., expression of activated Akt) may sensitize tumor cells to potent antitumor cytokines such as IL-2, IL-12, and IL-18.

More recently, attention has focused on the potential therapeutic role for inhibitors of proteasome function. Proteasomes play a key role in regulating the turnover of proteins that have been targeted for degradation by ubiquitination, including proteins that modulate cell cycle progression, survival, and apoptosis (9–11). More specifically, proteasome substrates include cyclin-dependent kinases such as p27 (8, 11, 17) and p21 (8, 11, 17), death receptor pathway components, including Fas/FasL (19), tumor suppressors such as p53 (17, 18), and several of the Bcl-2 family members, including Bid (55), Bcl-2 (8, 19), and Bcl-xL (8). In hematologic malignancies such as multiple myeloma, the proteasome inhibitor bortezomib can inhibit phosphorylation of Akt (23). Thus, the inhibition of the proteasome
function in rapidly dividing cancer cells may interfere with several of these prosurvival factors, halt cell cycle progression and induce apoptosis (8, 11). Bortezomib is a specific inhibitor of the 26S proteasome subunit (10) and has demonstrated potent antitumor activity against malignant cell types including multiple myeloma (11, 19, 23) and glioblastoma (8), as well as breast and lung (16), colorectal (17), and prostate carcinoma (15, 56) among others. Bortezomib was approved recently by the FDA for the management of patients with multiple myeloma (12) and is under active investigation for use in the treatment of various solid tumors when used alone or in combination with radiation and cytotoxic chemotherapy among other modalities (16, 17, 21, 24). More recently, a phase 1 investigation of single-agent bortezomib in children with advanced solid tumors was reported (57), although there is no specific information available to date regarding the efficacy of bortezomib in patients with neuroblastoma. In addition, there is no information in the existing literature regarding the antitumor activity of bortezomib in preclinical models of murine neuroblastoma.

The present studies were designed to investigate whether bortezomib possesses antitumor activity against neuroblastoma tumors in vitro or in vivo using a syngeneic model of transplantable murine neuroblastoma (TBJ/Neuro-2a). We provide the first reported evidence that bortezomib can mediate antitumor effects against murine neuroblastoma tumors, as evidenced by inhibition of proliferation, loss of mitochondrial membrane potential and induction of apoptosis even at low nanomolar concentrations of bortezomib in vitro. Furthermore, we have demonstrated for the first time that bortezomib can inhibit phosphorylation (activation) of Akt at Ser-473 within murine neuroblastoma tumor cells, and that inhibition of Akt phosphorylation by bortezomib occurs in conjunction with activation of Bid, a proapoptotic Bcl-2 family member that is normally suppressed by activated Akt (31, 32). Full-length Bid is localized in the cytoplasm of cells as an inactive precursor (25). In response to appropriate stimuli, including engagement of death receptors, Bid is cleaved into an active truncated form (tBid) that is translocated to the mitochondria, induces cytochrome c release, and potentiates apoptosis. Previous studies have shown that tBid is ubiquitinated and degraded by the 26S proteasome and that tBid-induced apoptosis can be significantly amplified in HeLa cells using the proteasome-specific inhibitor MG-132 (55). These findings suggest that, in addition to inhibition of Akt phosphorylation, stabilization of tBid by bortezomib could enhance the mitochondrial pathway of apoptosis in neuroblastoma tumor cells.

We also have shown for the first time that bortezomib can up-regulate the expression of receptors for both IFN-γ and TNF-α on TBJ neuroblastoma cells and sensitize these cells to apoptosis induced by IFN-γ plus TNF-α. In contrast, bortezomib did not increase surface expression of either Fas or TRAIL-R2 in TBJ cells, and they remain resistant to FasL or TRAIL-induced apoptosis. In that previous studies have shown that PS-341 can sensitize other tumor cell types to undergo TRAIL-mediated apoptosis (58), it appears that, depending on the setting, bortezomib may sensitize target cells to undergo apoptosis induced by several distinct death ligands. Bortezomib also sensitizes murine endothelial cells (EOMA) to IFN-γ plus TNF-α-mediated apoptosis in vitro, suggesting that in the context of immunotherapy, bortezomib could sensitize both the tumor and endothelial cell compartments to death ligand-mediated apoptosis. As predicted by these observations, administration of bortezomib markedly enhanced the antitumor activity of IFN-γ-TNF-α-inducing cytokines, including IL-2 and IL-12, in mice bearing primary and/or metastatic murine neuroblastoma tumors. Further, particularly in the case of combined administration of bortezomib and IL-12, these effects were achieved using a much lower dose-intensity of IL-12 than is required to achieve optimal therapeutic effects using other IL-12-based regimens. The ability of bortezomib to potentiate the effects of cytokine treatment for metastatic disease may be particularly relevant for neuroblastoma, where the outcome of patients with widespread metastases is very poor.

Collectively, these studies provide novel insight into the potential role of the proteasome as a therapeutic target for neuroblastoma, and describe a unique strategy using bortezomib to potentiate the proapoptotic and antitumor efficacy of immunotherapeutic cytokines. Because bortezomib and IL-2 are both already approved for use as single agents by the FDA, clinical trials may be considered to investigate the safety and efficacy of this combination in patients with advanced solid tumors, including neuroblastoma.

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Disclosures
The U.S. government has filed a patent application related to the subject matter of this manuscript, with J. M. Wigginton, T. Khan, and T. J. Sayers as coinventors.

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