Bortezomib Sensitizes Malignant Human Glioma Cells to TRAIL, Mediated by Inhibition of the NF-κB Signaling Pathway

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Abstract

Previous studies have shown that the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has significant apoptosis-inducing activity in some glioma cell lines, although many lines are either moderately or completely resistant, which has limited the therapeutic applicability of this agent. Because our recent studies showed that inhibition of proteasomal function may be independently active as an apoptosis-inducing stimulus in these tumors, we investigated the sensitivity of a panel of glioma cell lines (U87, T98G, U373, A172, LN18, LN229, LNZ308, and LNZ428) to TRAIL alone and in combination with the proteasome inhibitor bortezomib. Analysis of these cell lines revealed marked differences in their sensitivity to these treatments, with two (LNZ308 and U373) of the eight cell lines revealing no significant induction of cell death in response to TRAIL alone. No correlation was found between sensitivity of cells to TRAIL and expression of TRAIL receptors DR4, DR5, and decoy receptor DcR1, caspase 8, apoptosis inhibitory proteins XIAP, survivin, Mcl-1, Bcl-2, Bcl-Xl, and cFLIP. However, TRAIL-resistant cell lines exhibited a high level of basal NF-κB activity. Bortezomib was capable of potentiating TRAIL-induced apoptosis in TRAIL-resistant cells in a caspase-dependent fashion. Bortezomib abolished p65/NF-κB DNA-binding activity, supporting the hypothesis that inhibition of the NF-κB pathway is critical for the enhancement of TRAIL sensitization in glioma cells. Moreover, knockdown of p65/NF-κB by shRNA also enhanced TRAIL-induced apoptosis, indicating that p65/NF-κB may be important in mediating TRAIL sensitivity and the effect of bortezomib in promoting TRAIL sensitization and apoptosis induction. Mol Cancer Ther; 10(1); 198–208. ©2011 AACR.

Introduction

Glioblastomas are highly invasive primary tumors with a poor prognosis despite current surgical, radiation and chemotherapy approaches (1). Targeted therapies have failed to offer a long-term survival benefit in contrast to the improvements in outcome that have been achieved with new treatment approaches in many other cancer types. Genetic heterogeneity and a complex molecular pathology contribute to this lack of success, which highlights the need for novel therapies that target signaling pathways that underlie abnormal cellular growth. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor/death receptor (DR) gene superfamily, is a promising anticancer agent because it induces apoptosis preferentially in cancer cells (2). TRAIL can bind to 4 plasma membrane receptors and 1 soluble receptor, that is, TRAIL-R1 (DR4), TRAIL-R2 (DR5/KILLER), TRAIL-R3 [decoy receptor (DcR) 1], TRAIL-R4 (DcR2), and osteoprotegerin (3). TRAIL ligation with its receptors leads to formation of the death-inducing signaling complex (DISC), which recruits the adaptor molecule, FADD, and activates caspases-8 and -10. These activated caspases cleave and activate caspase-3, which in turn cleaves substrates that commit cells to undergo apoptosis (4). Alternatively, caspase-8 can cleave the proapoptotic Bcl-2 family member Bid, which translocates to the mitochondria and promotes mitochondrial dysfunction leading to apoptosis. This amplifies the apoptotic signal following death receptor activation (5).

TRAIL is a prime candidate for cancer therapy because it predominantly kills cancer cells while sparing normal cells. This tumor-selective cytotoxicity has been shown for glioma cells in comparison to nontransformed astrocytes in vitro (6). However, many glioma cell lines are resistant to TRAIL-induced apoptosis. The exact mechanism responsible for the resistance is not clear. Although the cell surface expression of DR4 or DR5 is required for TRAIL-induced apoptosis, tumor cells expressing these death receptors are not always sensitive to TRAIL due to intracellular aberrant activation of survival pathways (7). Recent reports suggest that TRAIL resistance may result...
from a combination of increased Akt activity (8), over-expression of Bcl-2 family members and other antiapoptotic molecules (9), and deficient expression of caspases. Accordingly, TRAIL alone may not be sufficient to efficiently activate apoptosis in many solid tumors, including gliomas (8, 10). Although resistance of some cancer cells to TRAIL can be reversed by treatment with protein synthesis inhibitors (11) or chemotherapeutic agents (12), these approaches have not been successful in more highly resistant glioma cell lines. Hence, much effort has been directed to identify new approaches to improve the efficacy of TRAIL-based apoptosis-inducing therapy.

In this regard, an siRNA-based screen by our group showed that inhibition of proteasome function was a particularly potent independent strategy for apoptosis promotion in gliomas (13). Proteasome inhibitors represent a promising novel class of anticancer agents (14) that is already in clinical use, as bortezomib (PS-341/Velcade) has been approved for the treatment of multiple myeloma (15). Moreover, PIs have been observed to induce apoptosis in solid tumors, such as lung and prostate cancer (16, 17), and several reports have shown that the combination of bortezomib and TRAIL overcomes the resistance to TRAIL in various types of cancer (18–22), although the primary mechanistic basis for this effect remains uncertain.

To address the potential contributors to TRAIL sensitivity in gliomas, we profiled the cytotoxic effects of this agent on 8 malignant human glioma cell lines, showing variable responses, with some cell lines being extremely sensitive and others highly resistant. We showed that this differential sensitivity correlated with activation status of NF-kB. In addition, bortezomib exhibited potent anti-glioma activity and dramatically sensitized even highly resistant glioma cells to TRAIL cytotoxicity, suggesting that this may be a promising combination strategy for glioma therapeutics.

Materials and Methods

Cell lines

The established malignant glioma cell lines U87, T98G, U373, LN229, and A172 were obtained from the American Type Culture Collection. LN18, LNZ428, and LNZ308 were generously provided by Dr. Nicolas de Tribolet. U87, T98G, and U373 were cultured in growth medium composed of minimum essential medium; LN18, LN229, A172, LNZ428, and LNZ308 were cultured in α-minimal essential medium. All growth media contained 10% fetal calf serum, l-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. Human astrocytes (HA) and human cerebellar astrocytes (HAC) were obtained from ScienCell Research Laboratories. Human umbilical vein endothelial cells (HUVEC) were purchased from Cell Applications Inc. Astrocytes and HUVEC growth media were obtained from respective vendors.

Reagents

Soluble human recombinant SuperKillerTRAIL (referred as TRAIL in this manuscript) was purchased from Enzo Biochemicals (Enzo Life Sciences), diluted and stored in KillerTRAIL storage and dilution buffer (Enzo Life Sciences). Caspase inhibitors (Z-IETD-fmk, Z-LEHD-fmk, Z-DEVD-fmk, and Z-VAD-fmk) were purchased from R & D Systems. Bortezomib was purchased from Chemie Tek.

Cell proliferation and cytotoxicity assay

Cells (5 × 10^3 per well) were plated in 96-well microtiter plates (Costar) in 100 μL of growth medium, and after overnight attachment, were exposed for 3 days to a range of concentrations of inhibitors, alone and in combination. Control cells received vehicle alone (DMSO). After the treatment interval, cells were washed in inhibitor-free medium, and the number of viable cells was determined using a colorimetric cell proliferation assay (CellTiter96 Aqueous NonRadioactive Cell Proliferation Assay; Promega). All studies were conducted in triplicate and repeated at least 3 times independently. Morphological changes such as cell shrinkage, rounding, and membrane blebbing were evaluated by microscopic inspection of cells. Images were taken using an Olympus FluoView 1000 microscope. Images were assembled using Adobe Photoshop CS2 software (Adobe Systems).

Clonogenic growth assay

The effect of different inhibitor concentrations on cell viability was also assessed using a clonogenic assay. For this analysis, 250 cells were plated in 6-well trays in growth medium, and after overnight attachment, cells were exposed to selected inhibitor concentrations or vehicle for 1 day. Cells were then washed with inhibitor-free medium and allowed to grow for 2 weeks under inhibitor-free conditions. Cells were then fixed and stained according to the manufacturer’s protocol (Hema 3 Manual Staining Systems; Fisher Scientific). After staining, 6-well plates were scanned and images were assembled using Adobe Photoshop CS2 software (Adobe Systems).

Annexin V apoptosis assay

Apoptosis induction in control (DMSO-treated) or inhibitor-treated cells was assayed by the detection of membrane externalization of phosphatidylserine with Annexin V-FITC conjugate using an Annexin V assay kit according to the manufacturer’s protocol (Invitrogen). In brief, 2 × 10^5 cells were harvested at various intervals after treatment and washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 200 μL of binding buffer. Annexin V-FITC and 1 μg/mL propidium iodide were added and cells were incubated for 15 minutes in a dark environment. The reaction was stopped by adding 300 μL of 1× binding buffer, and labeling was analyzed by flow cytometry with a FACSCalibur flow cytometer (BD Biosciences).
Antibodies and Western blot analysis

The following antibodies were used: p21 Waf1/ Cip 1 (#2946), Bim (#2819), Bcl-2 (#2872), Bcl-xL (#2764), DcR3 (#4758), DcR1 (#4756), FLIP (# 3210), XIAP (#2042), Survivin (#2808), FADD (#2782), Mcl-1 (#4572), Bik (#4592), Bid (#2002), Cleaved PARP (#9546), Cleaved Caspase 3 (#9664), Cleaved Caspase 8 (9496), DR3 (#3254), NF-κB p65 (#3034), IB-α (#4814), phospho-IB-α-Ser32/36 (#9246), β-Actin (#4970) were from Cell Signaling Technology Inc. DR4 (#IMG-141A) and DR5 (#IMG-120A) were from IMGENEX. Western blot analysis was performed as described previously (23). Scanning densitometry was done on Western blots using acquisition into Adobe Photoshop (Adobe Systems, Inc.) followed by image analysis (UN-SCAN-IT gel, version 6.1; Silk Scientific).

shRNA transfection

Four optimal 29mer-pRS-shRNA constructs were obtained from Origene. Sequences specific for human p65/NF-κB knockdown: GAT GAA GAC TTC TCC ATT GCC GAC AT (shRNA-1); GCT GTG TTC ACA GAC CGT CGA TTC GTC GA (shRNA-2); CCA CCA TCA AGA ATT GCG GAC AT (shRNA-1); GCT GTG TTC ACA GAC of time. Equal amounts of nuclear extracts were incubated at 15°C for 30 minutes with a biotinylated oligonucleotide containing the p65/NF-κB binding site, and then the samples were separated on 6.0% nondenaturing polyacrylamide gel in 0.5× TBE buffer for 90 minutes at 120 V at 4°C. To confirm specificity, NF-κB DNA binding was competitively blocked by an NF-κB cold probe. The gel contents were transferred onto Biodyne B membrane (Pall) for 45 minutes at 300 mA. The protein–DNA complexes were visualized after exposure to film.

DiOC6 labeling and detection of mitochondrial membrane depolarization

Mitochondrial membrane depolarization was measured as described (24). In brief, floating cells were collected, and attached cells were trypsinized and resuspended in PBS. Then the cells were loaded with 50 nmol/L 3,3′-dihexyloxacarbocyanine iodide (DiOC6; Invitrogen) at 37°C for 15 minutes. The positively charged DiOC6 accumulates in the intact mitochondria, whereas mitochondria with depolarized membranes accumulate less DiOC6. Cells were spun at 3,000 x g, and rinsed with PBS twice and resuspended in 1 mL of PBS. Fluorescence intensity was detected by flow cytometry (FACScan, Becton-Dickinson) and analyzed with the CellQuest software (Becton Dickinson). Percentage of cells with decreased fluorescence was determined.

Statistical analysis

Unless otherwise stated, data are expressed as mean ± SD. The significance of differences between experimental conditions was determined using a 2-tailed Student’s t test. Differences were considered significant at P values less than 0.05.

Results

Effect of soluble TRAIL on malignant human glioma cell lines

The cytotoxic effects of TRAIL were tested on a panel of 8 glioma cell lines. Cell proliferation assays showed that 3 of these cell lines, LN18, T98G, and LNZ428 were very sensitive (IC₅₀ ranging between 2.7 and 10.1 ng/mL); LN229, A172, and U87 revealed moderate sensitivity (IC₅₀ ranging between 31.3 and 41.8 ng/mL), whereas LNZ308 and U373 cells were resistant to TRAIL (IC₅₀ > 250 ng/mL; Fig. 1A, top). As shown in Fig. 1A (bottom), treatment with TRAIL for 24 hours induces cell death with characteristic apoptotic features, including cell detachment, shrinkage, and generation of apoptotic...
bodies as observed by phase contrast microscopy in TRAIL sensitive (LN18, T98G, and LNZ428) and moderately sensitive (A172, LN229, and U87) cells but not in TRAIL-resistant cell lines (LNZ308 and U373). Western blot (Fig. 2A, top) and clonogenic growth assay (Fig. 2A, bottom) studies revealed that TRAIL treatment resulted in significant PARP activation and cytotoxicity (colony formation) in TRAIL sensitive and moderately sensitive cells, but minimal or no activation in TRAIL-resistant cell lines.

To investigate the mechanisms controlling the resistance of glioma cells to the cytotoxic effect of TRAIL, a series of Western blot experiments were done to compare the expression of various components of the TRAIL signaling pathway among the 8 glioma cell lines. No correlation was found between the sensitivity of cells to TRAIL and the expression of TRAIL receptors DR4, DR5, or decoy receptor for TRAIL, DcR1, initiator caspase 8, and apoptosis inhibitory proteins XIAP, survivin, Mcl-1, Bcl-2, Bcl-xL, and cFLIP. All glioma cells showed high levels of Bcl-xL (Fig. 2B). Western blot analysis of LN18 (TRAIL-sensitive), U87 (moderately resistant), LNZ308, and U373 (TRAIL-resistant) cells did not show any significant changes in the levels of DR4, DR5, FLIP, and FADD after TRAIL treatment (Fig. 2C).

**Bortezomib sensitizes TRAIL-resistant glioma cells**

To investigate the potential of bortezomib to sensitize glioma cells to TRAIL cytotoxicity, we first examined the independent effect of bortezomib on cell proliferation in our panel of 8 established glioma cell lines by 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H tetrazolium (MTS) cell proliferation assay. Bortezomib resulted in a dose-dependent inhibition of cell proliferation with median effective concentrations of approximately 10 nmol/L (Supplementary Fig. 1, left). To confirm the specificity of bortezomib toward tumor cells, we compared the effect on normal cells (human astrocytes, HA; human cerebellar astrocytes, HAC; Human umbilical vein endothelial cells, HUVEC). At concentrations as low as 10 nmol/L, bortezomib eliminated 75% or more of the glioma cells, but had little or no effect on nonneoplastic cells (at 10 nmol/L), suggesting that bortezomib acts selectively against tumor cells compared with nonneoplastic cells (Supplementary Fig. 1, right).

We then examined the ability of bortezomib to promote an apoptotic effect of TRAIL in the TRAIL-resistant LNZ308 and U373 cell lines, at clinically relevant concentrations. Apoptotic cells were determined by flow cytometry after a 48-hour treatment. Our results showed that both LNZ308 (Supplementary Fig. 2, top) and U373 (Supplementary Fig. 2, bottom) cells were resistant to TRAIL, as TRAIL alone was unable to induce apoptosis. However, combining bortezomib at 5 nmol/L with TRAIL reversed the resistance and induced significant apoptosis in a dose-dependent manner starting with TRAIL concentrations as low as 1 ng/mL. In addition to short-term...
apoptosis assays, we also assessed the effect of bortezomib and TRAIL on long-term clonogenic survival of glioma cells. Colony-forming assays were used to determine whether inhibitor-treated cells reenter the cell cycle and divide. Cells were cultured in the presence of bortezomib (5 nmol/L) or in combination with indicated concentrations of TRAIL for 1 day. After 1 day, inhibitor was removed and then cells were cultured in inhibitor-free medium for 14
additional days. Neither bortezomib (Fig. 3, top) nor TRAIL (Fig. 2A, bottom) alone resulted in a significant reduction of viable cells. In contrast, combinatory treatment with bortezomib and TRAIL reduced cell viability significantly at day 14 after treatment (Fig. 3, top). In addition, simultaneous treatment with bortezomib and TRAIL resulted in a significant increase of cleaved caspase 3 and PARP, as determined by Western blot analysis (Fig. 3, bottom). These findings show that proteasomal inhibition cooperates with TRAIL to inhibit clonogenicity by preventing cells from reentering the cell cycle and dividing and by inducing apoptosis.

To understand the effect of bortezomib on TRAIL-resistant cell lines (U373 and LNZ308), we analyzed the involvement of caspase pathways using the chemical inhibitors IETD-fmk (caspase 8 inhibitor), LEHD-fmk (caspase 9 inhibitor), DEVD-fmk (caspase 3 inhibitor), and ZVAD-fmk (pan caspase inhibitor) in the culture media 4 hours prior to treatment with bortezomib and TRAIL. Apoptotic cells were determined by flow cytometry and Western immunoblot analysis after a 48-hour treatment. When LNZ308 and U373 cells were pretreated with caspase inhibitors, apoptosis induction was inhibited, indicating that the enhanced apoptosis induction by the combination of bortezomib and TRAIL was caspase-dependent (Supplementary Fig. 3).

Combination of TRAIL and bortezomib activates extrinsic and intrinsic apoptotic pathways

Loss of mitochondrial transmembrane potential is another indicator of mitochondrial dysfunction and insufficient activation of the extrinsic pathway has been implicated in contributing to TRAIL resistance at the mitochondrial level (25). Mitochondrial membrane depolarization and release of proapoptotic proteins (including cytochrome c, Smac/ DIABLO, AIF and HtrA2/Omi) have been implicated in both TRAIL and proteasome inhibitor-induced apoptosis (26). Because bortezomib induces loss of mitochondrial membrane potential and apoptosis (27), and mitochondrial changes are necessary for the activation of downstream caspases, we investigated the effect of bortezomib on mitochondrial membrane depolarization in the LNZ308 (Supplementary Fig. 4, top) and U373 (Supplementary Fig. 4, bottom) cell lines. Bortezomib induced mitochondrial membrane depolarization in a dose-dependent manner. However, combination of bortezomib and TRAIL induced strong increase in mitochondrial membrane depolarization. These results indicated that the synergistic interaction between the 2 stimuli is apparent at the level of mitochondria. Then, we evaluated the role of caspases on the effects on membrane depolarization observed following treatment with TRAIL and bortezomib, by assessing the effect of pretreatment with various caspase inhibitors. As shown in Supplementary Fig. 5, caspase inhibitors completely abolished the membrane depolarization induced by TRAIL and bortezomib. These results suggest that cotreatment of bortezomib and TRAIL affects the mitochondrial changes that typically occur during apoptosis.

Effects of bortezomib on death receptors on TRAIL-resistant cell lines

To further elucidate the molecular events underlying the observed enhancement of apoptosis by the combination of TRAIL and bortezomib in TRAIL-resistant cell lines (U373 and LNZ308), we examined expression of TRAIL receptors and TRAIL DISC proteins, including FADD and c-FLIP, in bortezomib-treated cells. LNZ308 and U373 cells were exposed to varying concentrations and durations of bortezomib treatment and Western blot analysis was done. As shown in Fig. 4A, bortezomib did not significantly alter the expression levels of DR4, DR5, FADD, and c-FLIP in U373 and LNZ308 cells. FADD, in particular, plays a key role in DISC formation and mediates TRAIL-induced apoptosis, and we found that the expression of FADD was minimally changed in the presence of bortezomib. We also analyzed the Bcl-2 family and proapoptotic protein levels. Bcl-2, Bcl-xl, Bik, Bid, and Bim levels were not changed significantly in cells exposed to bortezomib. In contrast, the expression of Mcl-1 and p21 was significantly enhanced by bortezomib treatment and Western blot analysis was done. As shown in Supplementary Fig. 5, caspase inhibitors completely abolished the membrane depolarization induced by TRAIL and bortezomib. These results suggest that cotreatment of bortezomib and TRAIL affects the mitochondrial changes that typically occur during apoptosis.
Bortezomib inhibits NF-κB DNA-binding activity and sensitizes glioma cells to TRAIL

It has been shown that bortezomib inhibits tumor cell growth by inhibiting NF-κB activation, particularly in tumors constitutively expressing this pivotal transcription factor (28). NF-κB activation in cancer cells contributes to resistance to TRAIL-induced apoptosis (29). Because NF-κB can modulate glioma cell survival and/or proliferation (30), we examined constitutive DNA-binding activity of NF-κB by EMSA in 8 established glioma cell lines. As shown in Fig. 5A, among the 8 established glioma cell lines, TRAIL-resistant cell lines (LNZ308 and U373) exhibited the highest levels of basal NF-κB activity. For further investigation, we selected LN18, U87, LNZ308, and U373 and assessed NF-κB DNA-binding activity. Dose response analysis revealed that bortezomib markedly inhibited NF-κB DNA-binding activity in all 4 cell lines (Fig. 5B). Because IB-α is a substrate of the ubiquitin-mediated proteasomal degradation pathway (31), bortezomib, a proteasome inhibitor, theoretically should stabilize IB-α protein through inhibition of proteasome activity. When examining the effects of bortezomib on IB-α degradation in 3 glioma cell lines, we surprisingly found that bortezomib decreased IB-α levels while increasing phospho IB-α levels in a dose-dependent manner which is consistent with the recent observation in multiple cancer cell lines (32). Furthermore, densitometric analysis showed that bortezomib effectively inhibited p65/NF-κB levels (Fig. 5C). That prompted us to investigate the role of TRAIL and bortezomib on p65/NF-κB DNA-binding activity when the cells were treated with TRAIL or bortezomib or the combination of both. The EMSA data showed that combination of TRAIL and bortezomib abolished p65/NF-κB DNA-binding activity (Fig. 5D) and these observations support the idea that inhibition of the NF-κB pathway is critical for the enhancement of TRAIL sensitization in glioma cells.

p65/NF-κB gene silencing induces TRAIL cytotoxicity in glioma cells

To directly define the effects of p65/NF-κB inhibition on TRAIL-sensitivity, we compared the levels of TRAIL-induced apoptosis in cells transiently transfected with a p65/NF-κB-specific shRNA construct in LNZ308 and U373 cells. We also examined the effects of p65/NF-κB shRNA knockdown on TRAIL sensitivity of LNZ308 and U373 cells. LNZ308 and U373 cells were transfected with p65/NF-κB shRNA, and 48 hours after transfection, Western blot analysis was done for p65/NF-κB expression and in parallel, cell viability was assessed by proliferation assay. Immunoblotting confirmed that the p65/NF-κB-specific shRNA construct efficiently reduced p65/NF-κB protein levels (40%–50% reduction compared with nontarget shRNA; Fig. 6A, top). As shown in Fig. 6A (bottom), p65/NF-κB knockdown significantly inhibited cell proliferation. To determine whether direct inhibition of p65/NF-κB enhanced TRAIL-sensitivity, LNZ308 and U373 cells were transiently transfected with nontarget shRNA or shRNA-1 against p65/NF-κB. Forty-eight hours post-transfection, cells were treated with varying concentrations of TRAIL for 72 hours, and cell proliferation was assessed by proliferation assay. As shown in Fig. 6B, TRAIL at the concentrations used had minimal or no effect on cell viability when added with nontarget shRNA. In contrast, strong inhibition of cell proliferation was observed when p65/NF-κB shRNA and TRAIL were combined. Taken together, p65/NF-κB knockdown synergized with TRAIL to promote apoptosis in LNZ308 and U373 cells.
Combined treatment with TRAIL and bortezomib does not induce cell death in nonneoplastic astrocytes and HUVECs

To characterize the potential interactions between bortezomib and TRAIL on normal cell viability, HA, HAC, and HUVEC were exposed to bortezomib or TRAIL or the combination of both and cell viability was assessed after 3 days by proliferation assay. The combination of bortezomib and TRAIL had little or no effect on HA proliferation (Supplementary Fig. 6, left). To investigate the molecular mechanisms for this differential sensitivity compared with glioma cells, HA, HAC, and HUVEC cells were exposed to bortezomib or TRAIL or the combination of both and a series of Western blot experiments were done to compare the expression of various components of the TRAIL signaling pathways. Unlike glioma cells (LNZ308 and U373), we found no expression of TRAIL receptors DR4 and DR5 or caspase activation. High expression of Bcl-xL was observed in HA, HAC, and HUVEC cells (Supplementary Fig. 6, right). Taken together, these results may explain the preferential cytotoxicity of bortezomib and TRAIL cotreatment in glioma cells, sparing astrocytes and endothelial cells.

Discussion

Proteasome inhibitors have attracted recent attention as potential antitumor agents but the mechanism by which proteasome inhibitors induce apoptosis is poorly understood. The ubiquitin/proteasome pathway represents the primary mechanism by which the bulk of cellular proteins in proliferating cells are degraded. By regulating proteolysis, and thus intracellular protein levels, the proteasome plays an important role in cell growth and apoptosis (33). A TRAIL-sensitizing effect of
bortezomib has been noted in various types of cancer (19, 22, 34), whereas the molecular mechanisms responsible for this synergy remain less clear-cut (35). Molecular targets responsible for the sensitizing effect of bortezomib on TRAIL-induced cell death include DR4 (18), DR5 (18, 20, 21), c-FLIP (20, 21), NF-κB (34, 36), p21 (19), and p27 (37). In addition, Bcl-2 family members also play a role in the combinational effect of bortezomib and TRAIL, including Bcl-2 (38), Bax (21), Bak (35), Bcl-xL (38), Bik (39), and Bim (40).

Although potential upregulation of TRAIL receptors has been suggested as one explanation for a TRAIL-sensitizing effect of bortezomib in various cancer cell lines (18, 20, 21), including some gliomas (13) we did not observe this effect in any of the 8 glioma cell lines we tested, suggesting that this mechanism may not be the primary contributor in these tumors. Because we observed a strong inverse association between NF-κB activation status and TRAIL response, we examined whether TRAIL sensitization by bortezomib reflects its effects on inhibiting the NF-κB signaling pathway (28, 41). Although inactivation of NF-κB has been suggested to play a major role in the antitumorigenic effect of bortezomib in multiple myeloma (42) and melanoma cells (43), inhibition of NF-κB is not required to sensitize hepatocellular carcinoma cells and lymphoma cells to apoptosis (44, 45), emphasizing the cancer type-specific effects of this agent.

Given the involvement of NF-κB activation in glioma cell resistance to TRAIL-induced apoptosis, we examined the effects of bortezomib on the levels of IκB-α, a key protein that regulates NF-κB activation. In the tested cell lines, we found that bortezomib decreased IκB-α level. The observation that bortezomib reduced IκB-α levels in human glioma cells is novel and these findings are consistent with previous studies showing that PS-341 as well as other proteasome inhibitors induce IκB-α degradation in endometrial carcinoma cell lines (46), HepG2 liver cancer cell line (47), multiple myeloma cells (48), and other tumor types (32). Recently, it has been shown that activated IκB kinase complex phosphorylates IκB-α on serines 32 and 36, leading to subsequent ubiquitination and proteasome-mediated degradation of IκB-α (32). Our results show that bortezomib induces IκB-α phosphorylation on serines 32 and 36 accompanied by reduction of IκB-α levels. This finding is in agreement with previous research.
work, showing that PS-341 and other proteasome inhibitors induce IB kinase-dependent IκB-α phosphorylation and degradation in cancer cell lines (48, 49). Our data suggest that bortezomib treatment not only induces IκB-α degradation, NF-κB inhibition, and independent apoptosis induction but also strongly enhances TRAIL-mediated cytotoxicity in malignant human glioma cell lines.

Our data also indicate that various targets of NF-κB may contribute to the apoptosis-modulating effects observed. For example, XIAP is an important antiapoptotic protein that confers resistance to TRAIL-cytotoxicity (50) and our results show striking downregulation of XIAP expression in response to bortezomib, and it is conceivable that a combination of NF-κB-mediated effects may be involved in the TRAIL sensitization seen in the resistant U373 and LNZ308 cell lines.

In summary, we report that inhibition of p65/NF-κB is an efficient strategy to broadly sensitize glioblastoma cells to TRAIL therapy, either via the death receptor (extrinsic) apoptosis pathway or via the mitochondrial (intrinsic) apoptosis pathway. The combination treatment is superior to single agent therapy in suppressing glioma cell viability. In addition, genetic silencing of p65/NF-κB shRNA significantly enhances TRAIL-induced apoptosis. From a therapeutic standpoint, our findings suggest that the levels of p65/NF-κB in glioma could have prognostic value for predicting the efficiency of TRAIL-based therapy. Moreover, our findings provide strong evidence that inhibition of p65/NF-κB is a promising approach to lower the threshold for apoptosis. In particular, bortezomib sensitizes glioma cells to TRAIL by inhibiting IB-α and NF-κB DNA binding activity. Taken together, our results provide a strong rationale for combining TRAIL with proteasome inhibition as a novel therapeutic combination for gliomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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