

Bortezomib Primes Glioblastoma, Including Glioblastoma Stem Cells, for TRAIL by Increasing tBid Stability and Mitochondrial Apoptosis

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Abstract

Purpose: Searching for novel approaches to sensitize glioblastoma for cell death, we investigated the proteasome inhibitor bortezomib.

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

Results: Bortezomib and TRAIL synergistically trigger cell death and reduce colony formation of glioblastoma cells (combination index < 0.1). Investigations into the underlying molecular mechanisms reveal that bortezomib and TRAIL act in concert to cause accumulation of tBid, the active cleavage product of Bid. Also, the stability of TRAIL-derived tBid markedly increases on proteasome inhibition. Notably, knockdown of Bid significantly decreases bortezomib- and TRAIL-mediated cell death. By comparison, silencing of Noxa, which is also upregulated by bortezomib, does not confer protection. Coinciding with tBid accumulation, the activation of Bax/Bak and loss of mitochondrial membrane potential are strongly increased in cotreated cells. Overexpression of Bcl-2 significantly reduces mitochondrial perturbations and cell death, underscoring the functional relevance of the mitochondrial pathway. In addition, bortezomib cooperates with TRAIL to reduce colony formation of glioblastoma cells, showing an effect on long-term survival. Of note, bortezomib profoundly enhances TRAIL-triggered cell death in primary cultured glioblastoma cells and in patient-derived glioblastoma stem cells, underlining the clinical relevance. Importantly, bortezomib cooperates with TRAIL to suppress tumor growth in an *in vivo* glioblastoma model.

Conclusion: These findings provide compelling evidence that the combination of bortezomib and TRAIL presents a promising novel strategy to trigger cell death in glioblastoma, including glioblastoma stem cells, which warrants further investigation. *Clin Cancer Res*; 17(12); 4019–30. ©2011 AACR.

Introduction

Glioblastoma is the most common primary brain tumor (1). Even though aggressive treatment protocols are used, therapy response is still very poor and median survival is only 14.6 months (2). Thus, it is crucial to develop alternative treatment strategies.

Human cancers often show inherent resistance to apoptosis (3). As most current treatment approaches, like

chemo- and radiotherapy, primarily act by inducing cell death in cancer cells (4), defects in cell death induction can lead to treatment resistance. Apoptotic cell death is mediated by two distinct pathways. The extrinsic pathway is initiated by stimulation of receptors of the tumor necrosis factor (TNF) superfamily, such as TNF-related apoptosis-inducing ligand (TRAIL) receptors resulting in the formation of the death-inducing signaling complex (DISC), activation of caspase-8 and subsequently effector caspases such as caspase-3 (5). The mitochondrial pathway is initiated by the permeabilization of the outer mitochondrial membrane, followed by the release of cytochrome c and other apoptosis-promoting factors from the mitochondrial intermembrane space into the cytosol, resulting in the formation of the cytochrome c/Apaf-1/caspase-9 containing apoptosome complex, which promotes the activation of caspase-9 and -3 (6). The release of apoptotic factors from mitochondria is tightly controlled by the action of pro- and antiapoptotic proteins of the Bcl-2 family (7). Both pathways are linked by the Bcl-2 family protein Bid, which initiates the mitochondrial pathway on its cleavage to tBid by caspases (7).

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Translational Relevance

Our study provides first evidence that the synergism of the proteasome inhibitor bortezomib and the death inducing ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) critically depends on tBid. Bortezomib and TRAIL cooperate to trigger cleavage of Bid into tBid and stabilization of tBid in the cytosol, thereby linking the extrinsic to the intrinsic apoptosis pathway. The translational relevance is underscored by parallel experiments in patient-derived primary glioblastoma cells and in an *in vivo* glioblastoma model that similarly show cooperative induction of cell death and inhibition of tumor growth. Importantly, our study shows for the first time that the combination of bortezomib plus TRAIL is active against patient-derived glioblastoma stem cells implicated in treatment resistance of glioblastoma. Thus, this preclinical evaluation of a rational combination of two new classes of targeted drugs provides the molecular basis for the design of future clinical studies of bortezomib plus TRAIL in glioblastoma and thus has important clinical implications.

One emerging treatment strategy in glioblastoma involves novel agents such as proteasome inhibitors (8). Bortezomib is a dipeptidyl boronic acid compound that reversibly blocks the proteolytic activity of the proteasome (9). Early clinical trials of bortezomib in glioblastoma show that it is well tolerated and safe, also in combination with temozolomide and radiation (10, 11). In addition, proapoptotic receptor agonists targeting TRAIL receptors represent promising therapeutics in oncology given their ability to trigger the cell's intrinsic death program preferentially in cancer versus normal cells (12, 13). However, primary or acquired resistance of many human cancers toward TRAIL requires alternative strategies to restore TRAIL sensitivity. Searching for strategies to overcome apoptosis resistance in glioblastoma, we investigated the molecular mechanism and therapeutic potential of the proteasome inhibitor bortezomib in combination with TRAIL in glioblastoma.

Materials and Methods

Cell culture and chemicals

Glioblastoma cell lines were obtained from the American Type Culture Collection (Manassas) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Inc.) supplemented with 10% fetal calf serum (FCS), 1 mmol/L glutamine and 1% penicillin/streptomycin (all from Biochrom). Glioblastoma stem cells were isolated and cultured as previously described (14). Bortezomib was obtained from Janssen-Cilag (Neuss), TRAIL from R&D Systems (Wiesbaden) and N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) from Bachem (Heidelberg). Other chemicals were purchased by Sigma (Deisenhofen) unless otherwise noted.

Short interfering RNA transfection

U87MG cells were transfected with 60 pmol of Bid short interfering RNA (siRNA; HSS141377, Invitrogen) or negative universal control (Invitrogen) using TransMessenger transfection reagent (Qiagen). Transfection medium was replaced by complete medium after 3.5 hours. Transfection was repeated the next day. Following the second transfection, cells were incubated for 72 hours before being treated with bortezomib and TRAIL.

Retroviral transduction

Retroviral transduction was carried out as previously described (15). Briefly, PT-67 producer cells (BD Biosciences) were transfected with pRetroSuper empty vector, pRetroSuper expressing nontargeting control short hairpin RNA (shRNA; sequence: GATCATGTAGATACGCTCA) or Noxa shRNA (ref. 16; sequence: GTAATTATGACACATTTC), pMSCV empty vector (Clontech) or pMSCV expressing murine Bcl-2 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Glioblastoma cells were transduced by spin transduction and selected with puromycin (Clontech) or blasticidin (Invitrogen).

Determination of cell death, viability, and clonogenic assay

Cell death was determined by fluorescence-activated cell-sorting (FACScan, Becton Dickinson) analysis of DNA fragmentation of propidium iodide-stained nuclei as described previously (17). Briefly, cells were harvested, washed with PBS and resuspended in hypotonic buffer containing 50 µg/ml propidium iodide, 0.1% sodium citrate, and 0.4% triton-X 100. The amount of hypodiploid DNA (sub-G₁ fraction) was determined by FACS analysis. Specific cell death was calculated using the following formula as described previously (18): $[(\text{observed cell death} - \text{spontaneous cell death}) * 100] / [(100 - \text{spontaneous cell death})]$. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche). For clonogenic assay, T98G cells were seeded as single cells in 6-well plates for 24 hours, treated for 4 hours with 30 nmol/L bortezomib before 2.5 ng/ml TRAIL was added for 6 hours. Then, drug-free medium was supplied and colony formation was assessed after 14 days by crystal staining (0.75% crystal violet, 50% ethanol, 0.25% NaCl, 1.57% formaldehyde).

Western blotting analysis

Western blotting analysis was carried out as described previously (19), using the following antibodies: mouse anti-Bmf, mouse anti-caspase-8, mouse anti-cFLIP, mouse anti-HtrA2/Omi, and mouse anti-Noxa (1:1000; Alexis Biochemicals), rabbit anti-Bak, mouse anti-Bcl-2 and rabbit anti-caspase-9 (1:1000; BD Pharmingen), rabbit anti-Bcl-X_L, mouse anti-FADD, mouse anti-Smac and mouse anti-XIAP (clone 28; 1:1000; BD Transduction Laboratories), rabbit anti-TRAIL-R2 (1:500;

Chemicon), rabbit anti-Bid, rabbit anti-Bim, and rabbit anti-caspase-3 (1:1000; Cell Signaling), rabbit anti-cIAP2 (1:1000; Epitomics), goat anti-cIAP1 and rabbit anti-Survivin (1:1000; R&D Systems), goat anti-Bik/NBK (1:1000; Santa Cruz Biotechnology), rabbit anti-Mcl-1 (1:5000; Stressgen), rabbit-anti-Puma (1:500; Sigma) and rabbit anti-BaxNT (1:5000; Upstate Biotechnology). Mouse anti- β -actin (1:10000; Sigma), mouse anti-GAPDH (1:5000; HyTest) or mouse anti- α -tubulin (1:3000; Calbiochem) were used as loading controls. Goat antimouse IgG, donkey anti-goat IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000; Santa Cruz Biotechnology) and goat antimouse IgG1, goat antimouse IgG2b, or rat antimouse kappa (clone 187.1; 1:5000; Southern Biotech) conjugated to horseradish peroxidase were used as secondary antibodies. Enhanced chemiluminescence was used for detection (Amersham Biosciences). Representative blots of at least two independent experiments are shown.

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). Cells were incubated with caspase-3 substrate for 30 minutes at 37°C and immediately analyzed by flow cytometry.

Immunoprecipitation of active Bax and Bak

Cells were lysed in CHAPS lysis buffer (10 mmol/L HEPES, pH 7.4; 150 mmol/L NaCl, and 1% CHAPS). 1 mg protein was incubated with 8 μ g mouse anti-Bax (6A7, Sigma) or 0.4 μ g mouse anti-Bak (Ab-1, Calbiochem) antibody in overnight rotation at 4°C, followed by the addition of 10 μ l pan-mouse IgG Dynabeads (Invitrogen), then incubated for 2 hours at 4°C, washed three times with CHAPS lysis buffer and analyzed by Western blotting analysis using BaxNT or Bak (BD Pharmingen) antibody, respectively.

Determination of mitochondrial membrane potential

To measure mitochondrial membrane potential, cells were incubated for 10 minutes with 100 ng/ml of tetramethylrhodamine methylester perchlorate (Sigma) at 37°C, trypsinized, washed, and immediately analyzed by flow cytometry.

Analysis of surface expression of TRAIL-R2

To determine surface receptor expression of TRAIL-R2, cells were incubated with 2 μ g mouse antihuman TRAIL-R2 monoclonal antibody (ApoTech Corporation) for 30 minutes at 4°C, washed in PBS containing 1% FCS, incubated with rabbit antimouse-F(ab')₂IgG/Biotin (BD Biosciences) for 20 minutes at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-PE (BD Biosciences) for 20 minutes at 4°C in the dark and analyzed by flow cytometry.

TRAIL DISC immunoprecipitation

Cells were incubated for 1 hour at 37°C either with 1 μ g/ml Flag-tagged TRAIL (Alexis) and/or bortezomib (100 nmol/L: U87MG; 30 nmol/L: T98G) or left untreated. After lysis using a buffer containing 50 mmol/L Tris-HCl, 1% (v/v) Triton-X 100, 150 mmol/L NaCl and protease inhibitor cocktail (Roche), Flag-tagged TRAIL was also added to the bortezomib-treated and untreated samples. The TRAIL-receptor-associated DISC was then immunoprecipitated, adding 1.25 μ g/ml mouse-anti Flag M2 antibody (Sigma) in overnight rotation at 4°C. Precipitated proteins were eluted using 10 μ l pan-mouse IgG Dynabeads (Invitrogen). Beads were washed three times with washing buffer I [50 mmol/L Tris-HCl, 500 mmol/L NaCl, and 1% (v/v) Igepal CA-630 (Nonidet P-40; Sigma)] and once with washing buffer II (25 mmol/L Tris-HCl). Samples were analyzed by Western blotting for expression of TRAIL-R2, cFLIP, FADD, and caspase-8.

Chorioallantoic membrane assay

Chorioallantoic membrane (CAM) assay was done as described previously (15). Briefly, 2 \times 10⁶ cells were resuspended in 10 μ l serum-free medium and 10 μ l Matrigel matrix (BD Biosciences) and implanted on the CAM of fertilized chicken eggs on day 8 of incubation. On day 9 tumors were treated with 20 ng/ml TRAIL and/or 50 nmol/L bortezomib daily for three days. Four days after seeding, tumors were sampled with the surrounding CAM, fixed in 4% paraformaldehyde, paraffin embedded, cut in 5 μ m sections and analyzed by immunohistochemistry using 1:1 hematoxyline and 0.5% eosin. Images were digitally recorded at a magnification of \times 2 with an AX70 microscope (Olympus), tumor areas were analyzed with ImageJ digital imaging software.

Statistical analysis

Statistical significance was assessed by Student's *t*-Test (two-tailed distribution, two-sample, unequal variance). Interaction between bortezomib and TRAIL was analyzed by the Combination index (CI) method using CalcuSyn software (Biosoft). Combination index (CI) < 0.9 indicates synergism, 0.9–1.1 additivity and > 1.1 antagonism.

Results

Bortezomib sensitizes glioblastoma cells to TRAIL-induced cell death

Initially, we optimized the experimental conditions for the cooperative interaction of bortezomib and TRAIL. Testing different treatment schedules, we found that preincubation with bortezomib for 4 hours before the addition of TRAIL led to the most pronounced cooperative effect (Supplementary Fig. S1). Therefore, we used this treatment schedule for all following experiments. Of note, bortezomib synergistically acted in concert with TRAIL to induce cell death in glioblastoma cells as calculated by combination index (Fig. 1A; Supplementary Table S1). Correspondingly, caspase-8, -3, and -9 were predominately cleaved in

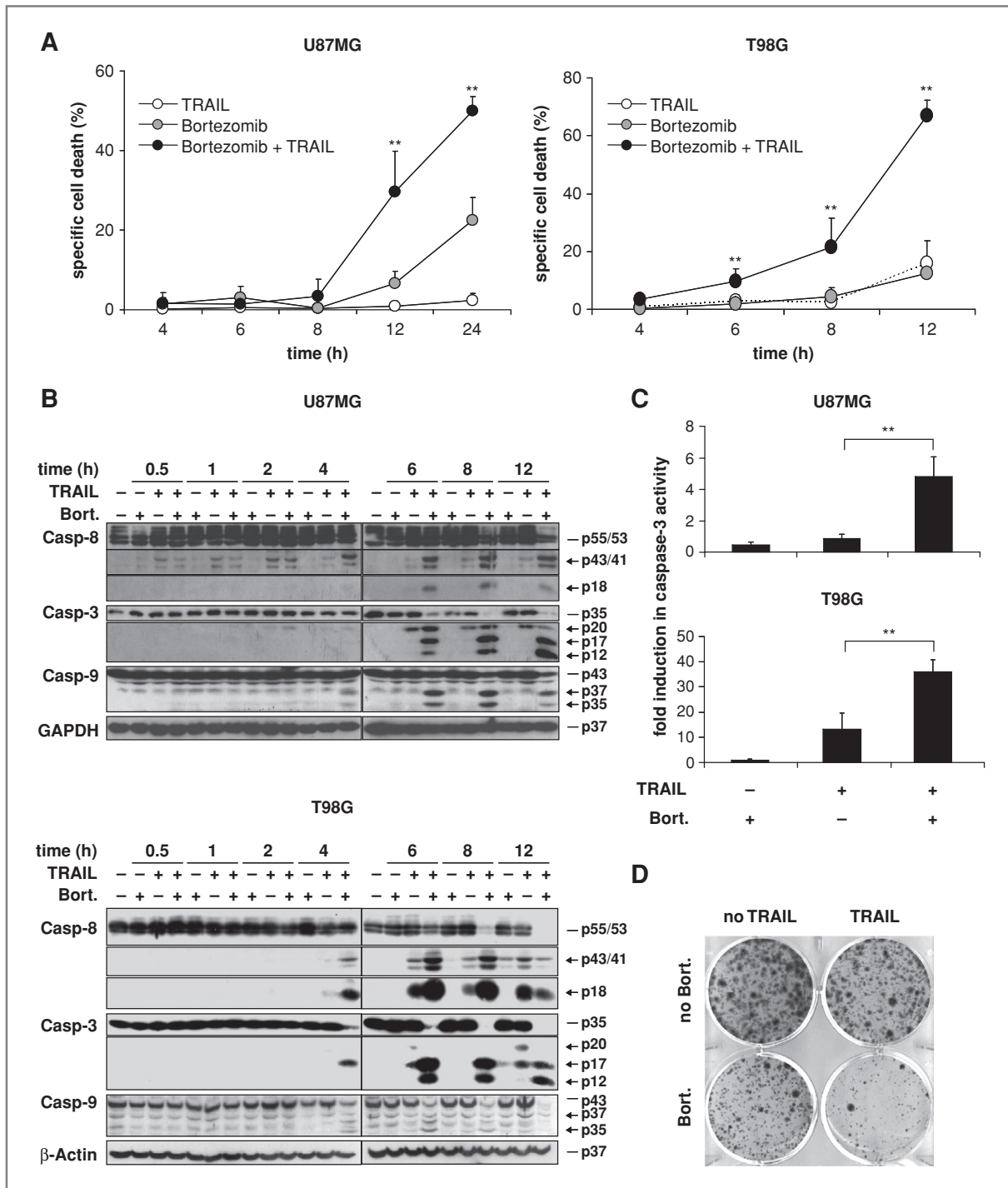


Figure 1. Bortezomib sensitizes glioblastoma cells to TRAIL-induced cell death. **A**, U87MG and T98G glioblastoma cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then TRAIL was added to a final concentration of 2.5 ng/ml for indicated times. Cell death was assessed by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Specific cell death is shown. Spontaneous cell death: U87MG: 7.66% +/- 1.03%; T98G: 5.98% +/- 0.93%. **B**, U87MG and T98G glioblastoma cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then TRAIL was added to a final concentration of 2.5 ng/ml and cleavage of caspase-8, -3, and -9 at the indicated time points and was assessed by Western blotting analysis. Cleavage fragments are indicated by arrows. GAPDH and β -actin served as loading controls. One representative experiment of 3 is shown. **C**, U87MG and T98G glioblastoma cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then TRAIL was added for 6 hours (U87MG) or 4 hours (T98G) at a concentration of 2.5 ng/ml. DEVD-specific caspase activity was determined by FACS analysis. Fold change in mean fluorescence intensity is shown. **D**, T98G glioblastoma cells were treated for 4 hours with 30 nmol/L bortezomib, then 2.5 ng/ml TRAIL was added for 4 hours. Colony formation was assessed at day 10 by crystal violet staining. **A** and **C**, data are means and SD of 3 independent experiments carried out in triplicate. **, $P < 0.001$.

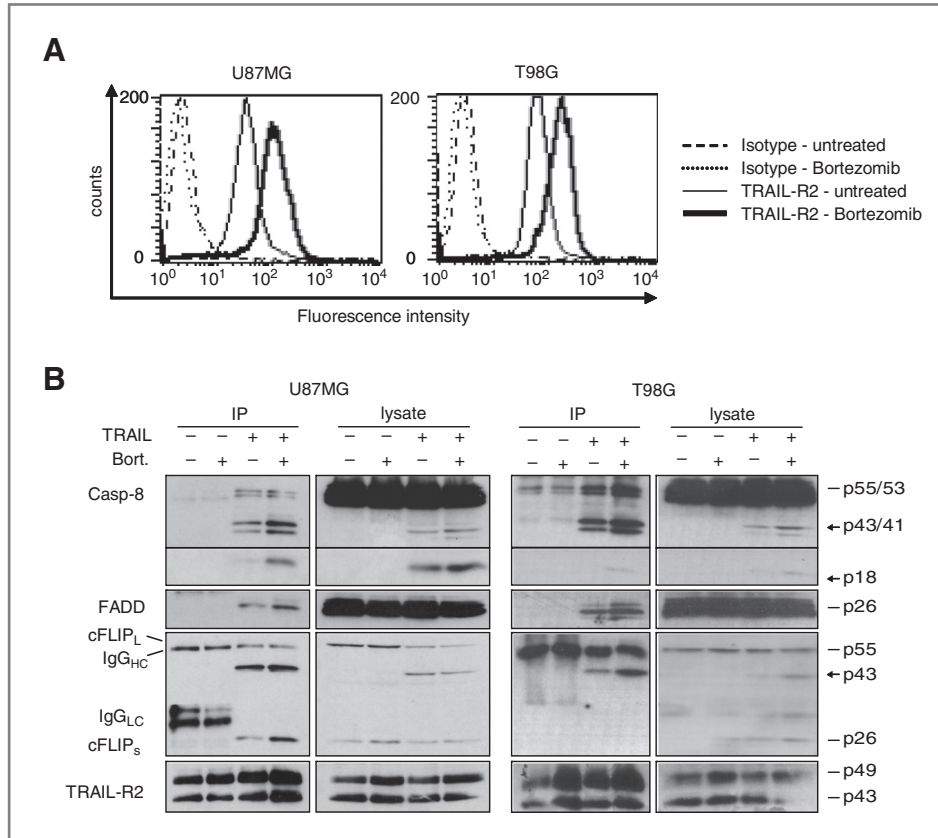


Figure 2. Effect of bortezomib on TRAIL-R2 surface expression and DISC formation. **A**, U87MG and T98G cells were treated for 10 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G). Surface expression of TRAIL-R2 was detected by fluorescence-conjugated antibody via flow cytometry (dashed line, untreated cells stained with isotype control; dotted line, bortezomib-treated cells stained with isotype control; thin gray line, untreated cells stained with TRAIL-R2 antibody; thick black line, bortezomib-treated cells stained with TRAIL-R2 antibody). One representative experiment out of 3 experiments carried out in triplicate is shown. **B**, U87MG and T98G cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then 1 μ g/ml Flag-tagged TRAIL was added for an additional 60 minutes. TRAIL-TRAIL-R complexes were immunoprecipitated as described in Materials and Methods. Expression of caspase-8, FADD, cFLIP_L, cFLIP_S, and TRAIL-R2 was analyzed by Western blotting. IgG_{LC}, IgG light chain; IgG heavy chain. Cleavage products are marked by arrows. One representative experiment of at least two is shown.

cotreated cells (Fig. 1B), which also showed significantly increased caspase-3 activity (Fig. 1C). Importantly, bortezomib acted in concert with TRAIL to reduce clonogenic growth of glioblastoma cells, showing that the combination treatment suppresses long-term survival (Fig. 1D).

Bortezomib increases TRAIL-R2 surface expression and formation of the TRAIL DISC

To gain insights into the molecular mechanisms, we investigated the contribution of the death receptor pathway in this model of apoptosis. Incubation with bortezomib significantly increased surface expression of TRAIL-R2 (Fig. 2A), while expression of TRAIL-R1, TRAIL-R3, and TRAIL-R4 were not altered (data not shown). Analysis of the DISC revealed that bortezomib enhanced the levels of caspase-8 and its cleavage fragments, of FADD, of the cFLIP_L cleavage fragment p43 and—in U87MG—of cFLIP_S on TRAIL treatment (Fig. 2B). The moderate increase in the recruitment and cleavage of caspase-8 at the TRAIL DISC might be explained by an increase in both proapoptotic (TRAIL-R2) as well as antiapoptotic (cFLIP) proteins by bortezomib.

Noxa is dispensable for bortezomib-mediated sensitization to TRAIL

Because caspase-9 cleavage coincides with full cleavage of caspase-3 (Fig. 1B), we speculated that activation of the mitochondrial pathway might contribute to the sensitization process. Therefore, we assessed activation of Bax and Bak as a crucial step to commit to mitochondrial apoptosis using conformation-specific antibodies. Importantly, activated Bax and Bak were predominately found in cotreated cells (Fig. 3A). This was accompanied by a significant increase in the loss of the mitochondrial membrane potential in cells cotreated with bortezomib and TRAIL (Fig. 3B).

Next, monitoring a panel of pro- and antiapoptotic proteins showed increased expression of Noxa, Mcl-1, and cFLIP_S on treatment with bortezomib (Fig. 3C). Accumulation of Bim_{EL} was observed in U87MG, but not in T98G cells (Fig. 3C, data not shown), suggesting that this was not a general mechanism. To test the functional relevance of bortezomib-mediated upregulation of Noxa we used RNA interference. Surprisingly,

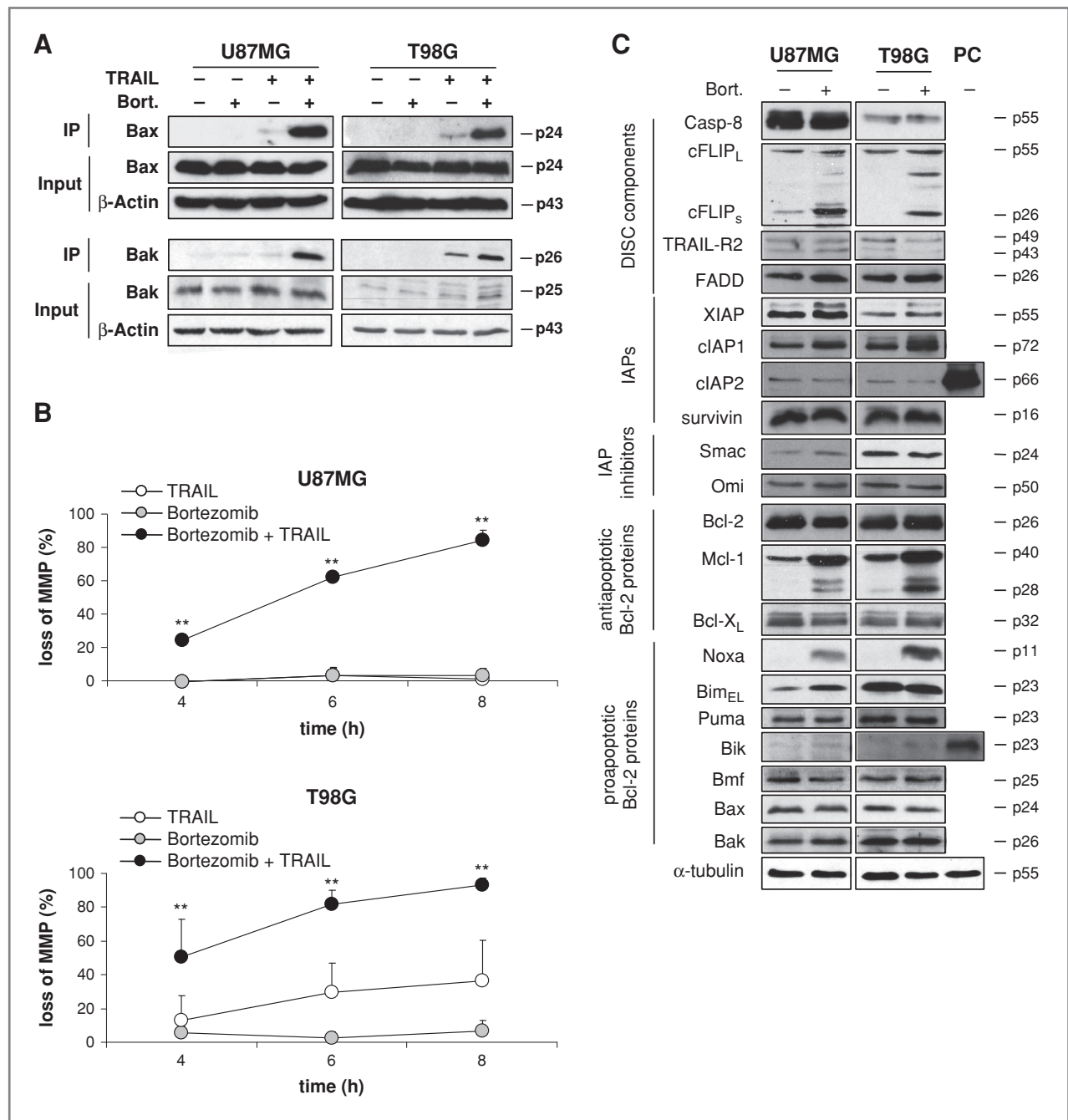


Figure 3. Bortezomib and TRAIL cooperate to induce mitochondrial perturbations. **A**, U87MG and T98G cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then 2.5 ng/ml TRAIL was added for 6 hours (U87MG) or 4 hours (T98G). Active Bax and Bak were immunoprecipitated by conformation-specific antibodies and detected by Western blotting. Bax, Bak, and β-actin expression in lysates served as loading control. One representative experiment of at least two is shown. **B**, U87MG and T98G cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then 2.5 ng/ml TRAIL was added and loss of mitochondrial transmembrane potential was determined by FACS analysis. Data are means and SD of three independent experiments carried out in triplicate. **, $P < 0.001$. **C**, U87MG (100 nmol/L) and T98G (30 nmol/L) were treated with bortezomib for 10 hours. Expression of apoptosis-regulatory proteins was determined by Western blotting. Jurkat T-cell acute lymphoblastic leukemia cells were used as positive control for Bik expression, EHEB chronic lymphocytic leukemia cells for cIAP-2 expression. α-Tubulin served as loading control. One representative experiment of at least two is shown.

knockdown of Noxa did not protect the cells from bortezomib- and TRAIL-induced cell death, while it significantly reduced cell death by camptothecin

(Supplementary Fig. S2), which was used as a positive control, because camptothecin-induced cell death has been reported to depend on Noxa (20). This shows that

Noxa is dispensable for bortezomib- and TRAIL-induced cell death.

Stabilization of tBid contributes to bortezomib-mediated sensitization to TRAIL

Next, we examined activation of Bid because it links the death receptor to the mitochondrial apoptosis pathway (7). Interestingly, we found that tBid, the cleaved form of Bid,

predominantly accumulated in cells treated with the combination of bortezomib and TRAIL (Fig. 4A). As tBid is prone to proteasomal degradation on its ubiquitination (21), we speculated that proteasome inhibition by bortezomib might prevent the degradation of newly generated tBid, leading to its accumulation. To investigate this, we first treated T98G cells for 12 hours with TRAIL to induce conversion of Bid to its truncated form tBid. After this stimulation, cells were

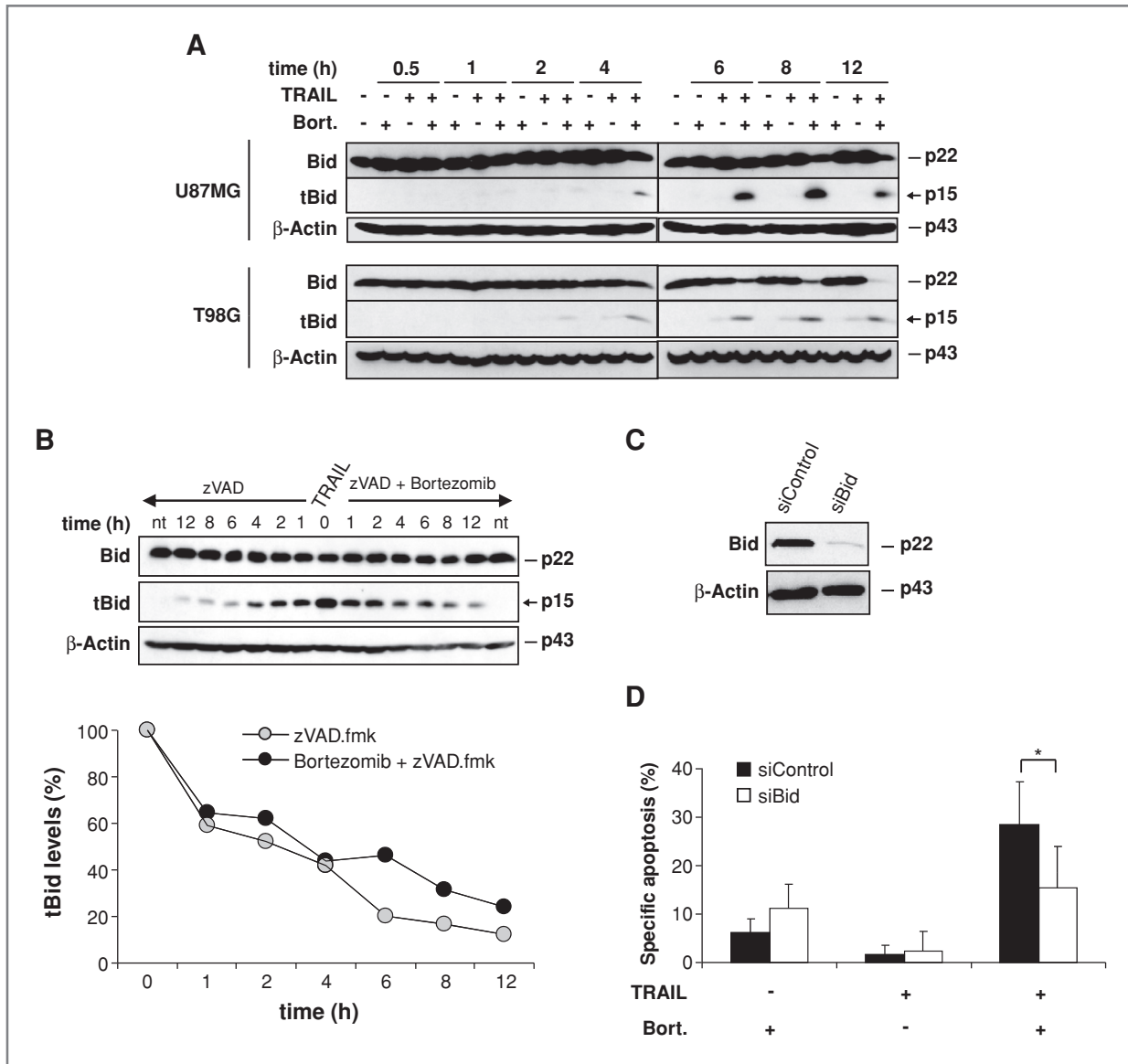


Figure 4. Bortezomib and TRAIL cooperate to cause accumulation of tBid. **A**, U87MG and T98G cells were treated for 4 hours with bortezomib (100 nmol/L: U87MG, 30 nmol/L: T98G), then TRAIL was added to a final concentration of 2.5 ng/ml. Cleavage of Bid to tBid was assessed at indicated times by Western blotting analysis. β -Actin served as loading control. One representative experiment of two is shown. **B**, T98G cells were treated for 12 hours with 2.5 ng/ml TRAIL. Then cells were washed three times to remove all residual TRAIL. Cells were then incubated with 20 μ mol/L zVAD in the presence or absence of 30 nmol/L bortezomib for indicated times. tBid protein expression was determined by Western blotting (top) and quantified by densitometry (bottom). Shown is the percentage of tBid levels relative to tBid expression at the end of the 12 hour-incubation period with TRAIL before addition of zVAD and bortezomib, which is set as 100%. One representative experiment is shown, similar results were obtained in three independent experiments. **C** and **D**, U87MG cells were transfected with either control siRNA (siControl) or Bid siRNA (siBid). **C**, 72 hours after transfection, expression of Bid was determined by Western blotting. **D**, cells were treated with 100 nmol/L bortezomib for 4 hours, then 2.5 ng/ml TRAIL was added for an additional 16 hours. Cell death was analyzed by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Specific cell death is shown. Spontaneous cell death: siControl: 8.69% \pm 2.26%; siBid: 11.25% \pm 2.53%. Data are means and SD of three independent experiments carried out in triplicate. *, $P < 0.05$.

washed to remove all remaining TRAIL. To prevent ongoing tBid conversion, e.g., by action of caspase-8, caspases were broadly inhibited using zVAD.fmk, which was added after removal of TRAIL. Therefore, no tBid is produced any longer, neither by ongoing TRAIL stimulation nor by continued caspase activity. tBid levels were then monitored over time in the presence or absence of bortezomib. Importantly, the addition of bortezomib delayed the decline of tBid (Fig. 4B). To investigate whether Bid is required for cell death induction by bortezomib and TRAIL, we knocked down Bid by RNAi (Fig. 4C). Of note, silencing of Bid significantly reduced cell death in cotreated cells (Fig. 4D). These results suggest that the stabilization of tBid contributes to the bortezomib-conferred sensitization to TRAIL-induced cell death.

Requirement of the mitochondrial pathway for bortezomib-mediated sensitization to TRAIL

To further investigate the importance of the mitochondrial pathway in this model of apoptosis, we ectopically expressed Bcl-2. Notably, overexpression of Bcl-2 significantly reduced cell death, Bax activation, loss of the mitochondrial membrane potential, and cleavage of caspase-9 and -3 on treatment with bortezomib and TRAIL (Fig. 5). These findings underline the relevance of mitochondria-mediated signaling events in bortezomib- and TRAIL-induced cell death.

Bortezomib cooperates with TRAIL to induce cell death in primary glioblastoma and glioblastoma stem cells and to suppress tumor growth *in vivo*

To underline the clinical relevance of our findings, we extended these studies to primary-cultured glioblastoma cells. Bortezomib profoundly enhanced TRAIL-induced loss of viability in several primary glioblastoma samples (Fig. 6A). Furthermore, we isolated glioblastoma stem cells from primary tumor samples, which were characterized by CD133 and Nestin staining and their ability to differentiate into the three different neuronal lineages (Supplementary Fig. S3). Importantly, bortezomib significantly enhanced TRAIL-induced cell death in glioblastoma stem cells derived from four distinct specimens (Fig. 6B), which was accompanied by increased caspase-3 cleavage in cotreated cells (Fig. 6C).

Finally, we tested the antitumor activity of bortezomib alone and in combination with TRAIL *in vivo* using the chorioallantoic membrane (CAM) model, an established *in vivo* tumor model (15, 22, 23). The CAM assay has previously been shown to serve as a suitable model for pre-clinical evaluation of anticancer agents (24). Glioblastoma cells were seeded on the CAM of chicken embryos, allowed to form tumors for 24 hours and treated for three days with TRAIL in the presence or absence of bortezomib. Importantly, bortezomib acted in concert with TRAIL to suppress glioblastoma growth *in vivo* (Fig. 6D). Together, this set of experiments shows that bortezomib sensitizes primary glioblastoma cells as well as glioblastoma stem cells for TRAIL-induced cell death and cooperates with TRAIL to reduce glioblastoma growth *in vivo*.

Discussion

In the present study we provide a compelling argument for the use of the proteasome inhibitor bortezomib to synergistically increase TRAIL sensitivity in glioblastoma by showing that bortezomib effectively sensitizes glioblastoma cell lines, primary glioblastoma cultures, and glioblastoma stem cells. Furthermore, we highlight a molecular mechanism not yet described for the combination of bortezomib and TRAIL (Fig. 7). We provide first evidence that bortezomib increases the stability of TRAIL-derived tBid. This finding is in line with work of Breitschopf and colleagues showing that tBid is rapidly ubiquitinated and subsequently degraded by the 26s proteasome (21). In the present study, bortezomib at nanomolar concentrations, which sensitize glioblastoma cells for TRAIL-induced cell death, markedly stabilizes tBid, which is generated on stimulation with TRAIL. This finding suggests that the combined action of TRAIL (leading to cleavage) and bortezomib (increasing tBid stability) on Bid cooperates to cause accumulation of tBid thereby linking the extrinsic to the intrinsic apoptosis pathway. The concerted action of bortezomib and TRAIL on the intrinsic apoptosis pathway is shown by activation of Bax and Bak, loss of mitochondrial membrane potential and caspase-9 cleavage predominantly in co-, but not in single-treated cells. The functional importance of Bid is underscored by knock-down experiments showing that Bid silencing significantly decreases cell death in cotreated cells. The key contribution of the mitochondrial pathway to TRAIL- and bortezomib-induced cell death is underlined by overexpression of Bcl-2, which significantly reduces cell death, loss of mitochondrial membrane potential and Bax activation.

While Noxa upregulation has been reported to be crucial for apoptosis induction, when bortezomib was used as a single agent (25–28), our data are the first to show that bortezomib-mediated accumulation of Noxa is in fact dispensable for TRAIL- and bortezomib-induced cell death in glioblastoma cells. This might be explained by concomitant accumulation of activated tBid on the combination of bortezomib with TRAIL. Also other BH-3 only proteins, such as Bim and Bik, have been implicated in TRAIL and bortezomib-mediated apoptosis (29). Bim is regulated by the Forkhead box O (FOXO) transcription factors, which are critically involved in apoptosis regulation (30). It has been shown that a number of apoptosis regulating genes are transcriptionally controlled by FOXO proteins, including Bim (31) and TRAIL (32). In addition, the stability of FOXO proteins is regulated by the proteasome (33) and it has been shown that bortezomib treatment leads to accumulation of Foxo3a and its transcriptional targets Bim and TRAIL in leukemia cells (34). We found a modest upregulation of Bim after bortezomib treatment in the U87MG cell line, whereas Bim levels remained unchanged in T98G cells. Because T98G cells were also sensitized to TRAIL by bortezomib, Bim upregulation is likely not a general mechanism of bortezomib-mediated sensitization to TRAIL in glioblastoma cells. Furthermore, Bik was not expressed in the glioblastoma cell lines investigated

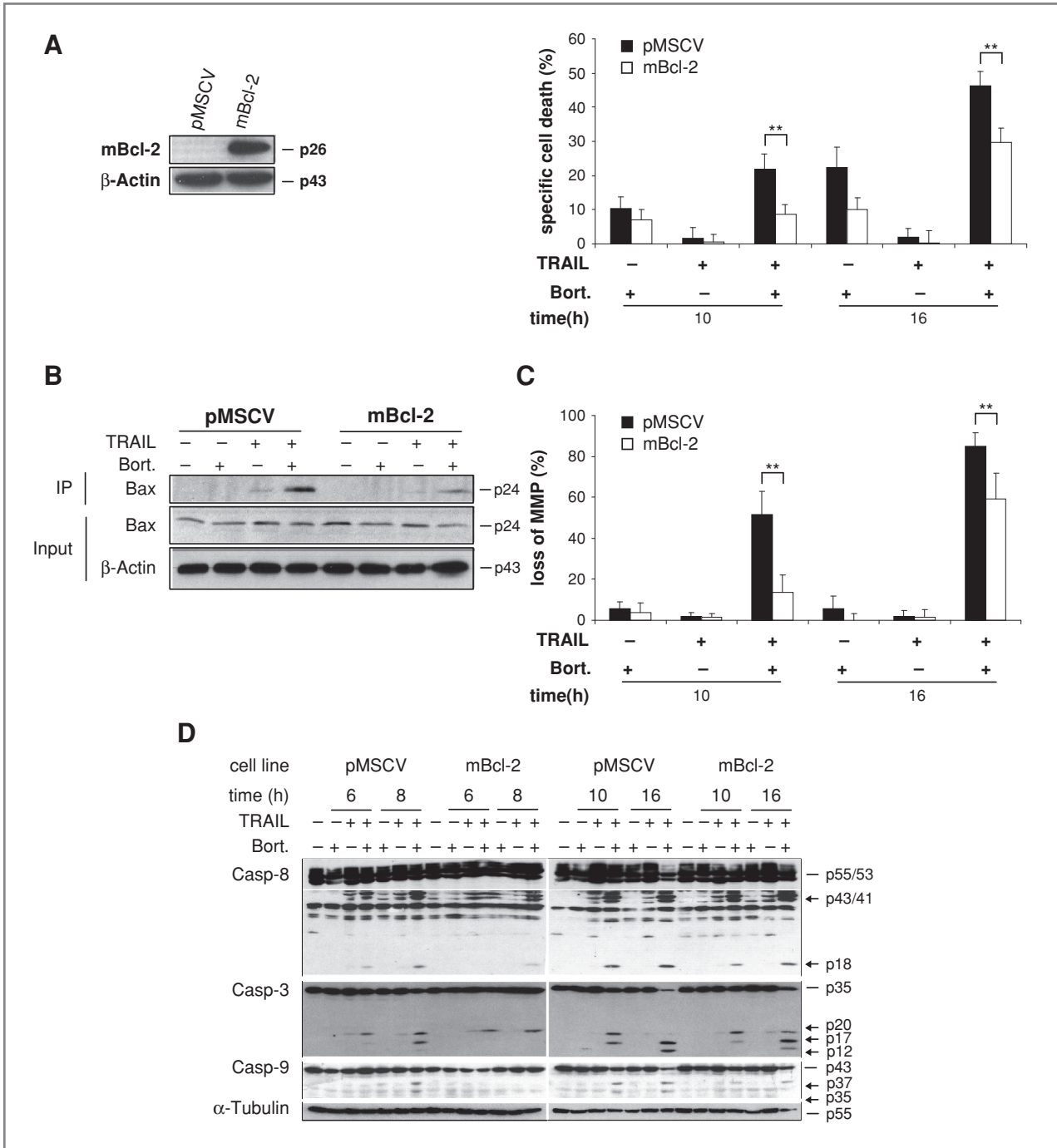


Figure 5. Bcl-2 overexpression delays bortezomib- and TRAIL-mediated cell death. U87MG cells were transduced with a vector expressing mouse Bcl-2 (mBcl-2) or empty vector (pMSCV). A, expression of mBcl-2 was determined by Western blotting (left). β-Actin served as loading control. In the right panel of A, cells were treated with 100 nmol/L bortezomib for 4 hours, then 2.5 ng/ml TRAIL was added for 10 and 16 hours. Cell death was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Specific cell death is shown. Spontaneous cell death: pMSCV: 9.59% +/- 1.42%; mBcl-2: 8.44% +/- 0.47%. Data are means and SD of three independent experiments carried out in triplicate. **, *P* < 0.001. B, cells were treated with 100 nmol/L bortezomib for 4 hours, then 2.5 ng TRAIL was added for 6 hours. Active Bax was immunoprecipitated by conformation-specific antibody and detected by Western blotting. Bax and β-actin expression in lysates served as loading control. One representative experiment of two is shown. C, cells were treated with 100 nmol/L bortezomib for 4 hours, then 2.5 ng/ml TRAIL was added for 10 and 16 hours. Loss of mitochondrial transmembrane potential was determined by FACS analysis. Data are means and SD of five independent experiments conducted in triplicate. **, *P* < 0.001. D, cells were treated with 100 nmol/L bortezomib for 4 hours, then 2.5 ng/ml TRAIL was added for indicated times. Expression of caspase-8, caspase-3, and caspase-9 was determined by Western blotting. Cleavage products are marked by arrows. α-Tubulin served as loading control. One representative experiment of two is shown.

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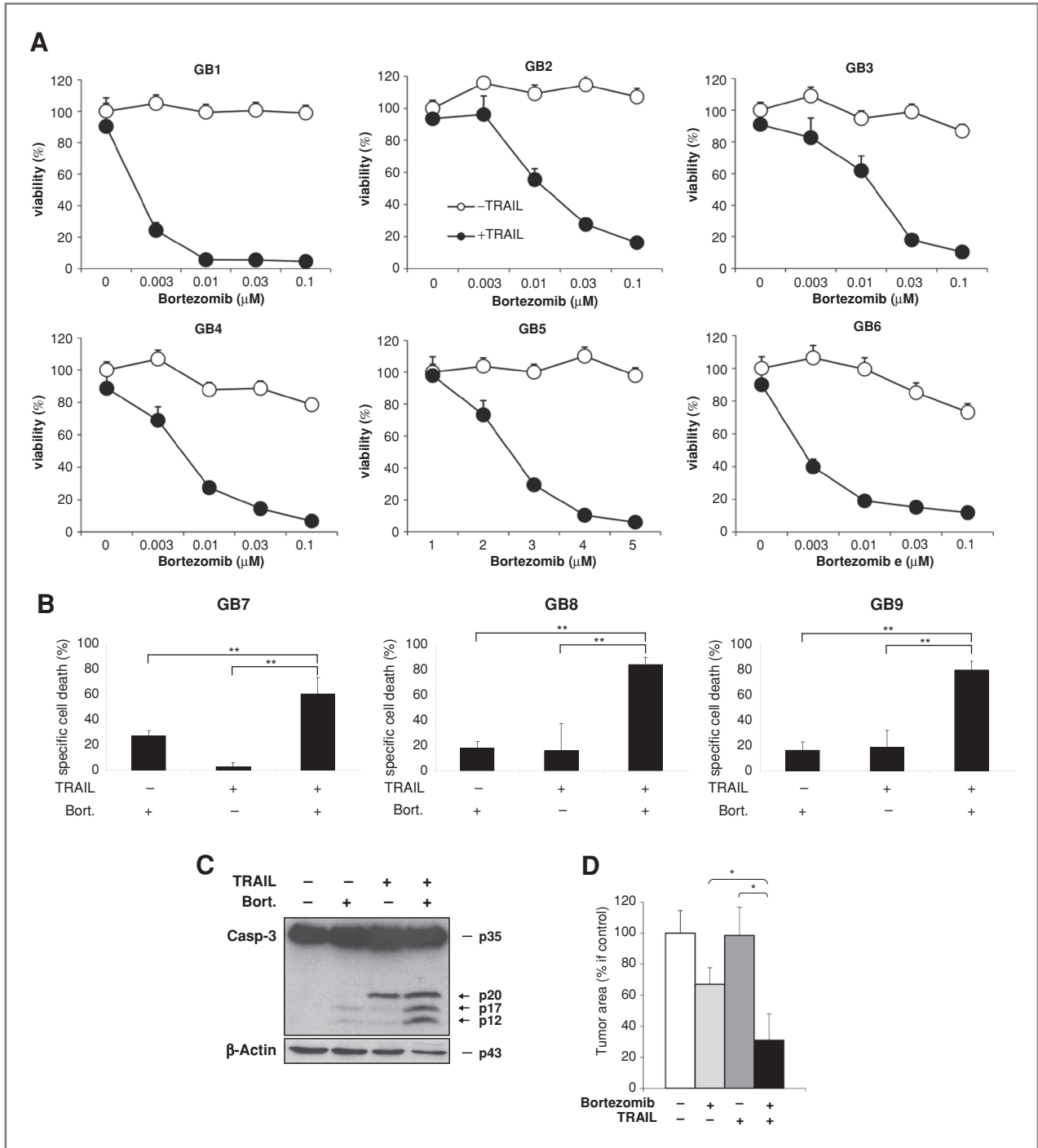


Figure 6. Bortezomib cooperates with TRAIL to induce cell death in primary glioblastoma cells and glioblastoma stem cells and to suppress tumor growth *in vivo*. **A**, primary glioblastoma cells derived from 6 different tumor specimens were treated for 4 hours with the indicated concentrations of bortezomib, then 10 ng/ml TRAIL was added for 24 hours and viability was assessed by MTT assay. Data are means and SD of 3 independent experiment carried out in triplicate. **B**, glioblastoma stem cells isolated from 3 different tumor specimens were treated for 4 hours with 10 nmol/L bortezomib, then 10 ng/ml TRAIL was added for an additional 24 hours. Cell death was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei. Specific cell death is shown. Spontaneous cell death: GB7: 20.19% \pm 7.97%; GB8: 10.45% \pm 2.27; GB9: 30.85% \pm 6.02%. Data are means and SD of 3 independent experiments carried out in duplicate. **, $P < 0.001$. **C**, glioblastoma stem cells were treated for 4 hours with 10 nmol/L bortezomib, then cells were stimulated for 16 hours with 10 ng/ml TRAIL. Cleavage of caspase-3 was analyzed by Western blotting. β -Actin served as loading control. **D**, U87MG glioblastoma cells were seeded on the CAM of chicken embryos, allowed to form tumors and treated with 30 nmol/L bortezomib and/or 10 ng/ml TRAIL. Tumor growth was analyzed using hematoxylin/eosin-stained paraffin sections of the CAM. Shown is tumor area as percentage of the untreated control group, error bars indicate mean \pm SD of 6 samples per group; *, $P < 0.05$. Similar results were obtained in 2 independent experiments.

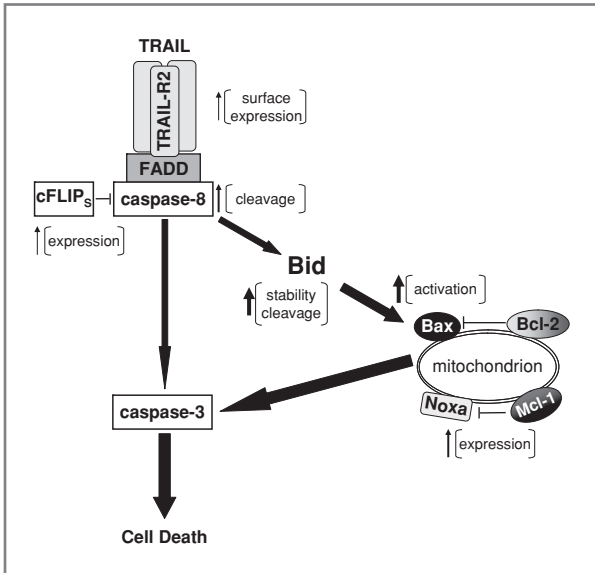


Figure 7. Scheme of the cooperation action of bortezomib and TRAIL. The combined action of TRAIL (leading to Bid cleavage) and bortezomib (increasing tBid stability) on Bid cooperates to cause accumulation of tBid thereby linking the extrinsic to the intrinsic apoptosis pathway and leading to enhanced Bax activation and mitochondria-driven cell death. Bcl-2 overexpression protects from bortezomib- and TRAIL-induced cell death underlining the importance of the mitochondrial pathway. Also, bortezomib causes accumulation of both Noxa and Mcl-1. At the TRAIL DISC, bortezomib concomitantly increases TRAIL-R2 surface expression and cFLIP_s, resulting in a slight increase in caspase-8 recruitment to the DISC. See text for more details.

suggesting that certain BH-3 only proteins may exert a more restricted function in a cell type-dependent manner in TRAIL- and bortezomib-induced cell death.

In earlier studies, it was suggested that bortezomib might predominately sensitize toward TRAIL by facilitating death receptor signaling via decreased recruitment of cFLIP_L and increased caspase-8 recruitment to the DISC (35, 36). Our data however, point to a minor role of the extrinsic apoptotic pathway in bortezomib- and TRAIL-induced cell death in glioblastoma. In line with earlier studies (37–39) we find increased TRAIL-R2 surface expression on bortezomib treatment. TRAIL receptor expression can be transcriptionally upregulated by the endoplasmic reticulum-stress related protein CHOP (40), by NFκB (41) and p53 (42). However, we do not detect any changes in the total amount of TRAIL-R2 protein in whole cell lysates, indicating that bortezomib may trigger increased TRAIL-R2 surface expression without detectable changes in total protein expression of TRAIL-R2 in glioblastoma cells. While bortezomib causes increased TRAIL-R2 expression, elevated levels of cFLIP_s are simultaneously detected in the DISC of cells

treated with bortezomib and TRAIL. As a net result, there is a moderate increase in caspase-8 or its active cleavage fragments in the TRAIL DISC, suggesting that these changes likely do not represent the key mechanism of sensitization.

The relevance of the study is highlighted by the following points: First, this preclinical study involves new targets and emerging treatment options for glioblastoma, i.e., the proteasome inhibitor bortezomib and the death receptor ligand TRAIL, and is based on strong mechanism-based hypotheses. Second, the clinical relevance of our data is confirmed by parallel experiments in clinical tumor material. Third, we provide first evidence that the combination of bortezomib plus TRAIL is also active against patient-derived glioblastoma stem cells. As glioblastoma stem cells have been implicated to contribute to glioblastoma progression due to their increased resistance to radio- and chemotherapy (43, 44), so therapeutic targeting of these cells will likely be critical to ensure treatment success. Finally, our data have important implications for the development of future therapies to increase the efficacy of current treatment protocols in glioblastoma. Because it has recently been shown that bortezomib is well tolerated, safe, and exerts some clinical activity in patients with recurrent glioblastoma (11), it might be feasible to translate this combination strategy of bortezomib plus TRAIL into a clinical setting in glioblastoma. In addition, proapoptotic TRAIL receptor agonists are in early clinical development for various solid tumors and hematologic malignancies (5). Taken together, this study provides convincing evidence that bortezomib in combination with TRAIL presents a promising novel strategy to trigger cell death pathways in glioblastoma including glioblastoma stem cells, which warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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