Cooperative Cytotoxicity of Proteasome Inhibitors and Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand in Chemoresistant Bcl-2-Overexpressing Cells

Alessio Nencioni,[1,2] Lucia Wille,[1] Giovanna Dal Bello,[2] Davide Boy,[2] Gabriella Cirmena,[2] Sebastian Wesselborg,[3] Claus Belka,[4] Peter Brossart,[5] Franco Patrone,[2] and Alberto Ballestrero[2]

Abstract

Purpose: Bcl-2 overexpression is frequently detected in lymphoid malignancies, being associated with poor prognosis and reduced response to therapy. Here, we evaluated whether Bcl-2 overexpression affects the cytotoxic activity of proteasome inhibitors taken alone or in association with conventional anticancer drugs or tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).

Experimental Design: Jurkat cells engineered to overexpress Bcl-2 were treated with proteasome inhibitors (MG132, epoxomicin, and bortezomib), anticancer drugs (etoposide and doxorubicin), TRAIL, or combinations of these compounds. Cell death and loss of mitochondrial transmembrane potential were detected by flow cytometry. Cytosolic relocalization of cytochrome *c* and SMAC/Diablo, caspase cleavage, and Bcl-2 and Mcl-1 levels were determined by immunoblotting. Nuclear factor-κB inhibition was done by retroviral transduction with a dominant-negative mutant of IκBα.

Results: Bcl-2 overexpression results in significant inhibition of apoptosis in response to proteasome inhibitors, antiblastics, and TRAIL. Addition of TRAIL to proteasome inhibitors results in a synergistic cytotoxic effect in Bcl-2-overexpressing cells, whereas this result is not reproduced by the combination of proteasome inhibitors with antiblastic drugs. Importantly, proteasome inhibitors plus TRAIL induce mitochondrial dysfunction irrespective of up-regulated Bcl-2. Bcl-2 cleavage to a fragment with putative proapoptotic activity and elimination of antiapoptotic Mcl-1 may both play a role in proteasome inhibitors-TRAIL cooperation. Conversely, nuclear factor-κB inhibition by proteasome inhibitors is per se insufficient to explain the observed synergy.

Conclusions: Combined proteasome inhibitors and TRAIL overcome the apoptotic threshold raised by Bcl-2 and may prove useful in the treatment of chemoresistant malignancies with up-regulated Bcl-2.

Bcl-2 is the founding member of a group of proteins (the Bcl-2 family) that control apoptosis at the mitochondrion level by regulating release of apoptotic factors into the cytosol (1, 2). Bcl-2 was first identified at the chromosomal breakpoint of t(14;18)-bearing human follicular lymphomas (3). Subsequent studies showed that high Bcl-2 expression is detectable in various hematologic malignancies and solid tumors (2, 4, 5). Here, Bcl-2 protein exerts its oncogenic role by preventing tumor cells from undergoing apoptosis due to enhanced activity of proliferation oncogenes, such as *myc* (6, 7). Besides, it confers resistance to radiation, chemotherapy, and hormonal therapy (2, 4, 5). Importantly, Bcl-2 up-regulation has been associated to poor prognosis and chemoresistance in diffuse large B-cell lymphoma, a neoplasm potentially curable with modern chemotherapy (8–10).

This evidence has led to the evaluation of novel agents to overcome chemoresistance associated to Bcl-2 up-regulation. Bcl-2 antisense oligonucleotides to shutdown Bcl-2 expression were shown to increase susceptibility to antiblastic agents in tumor cells of different histology and have yielded promising preliminary clinical results (11–14). Similarly, small-molecule antagonists targeting Bcl-2 have also recently emerged as effective Bcl-2 inhibitors in preclinical studies (15). Other compounds were reported to cooperate with anticancer drugs and γ-irradiation for the induction of apoptosis in Bcl-2-overexpressing tumor cell. These include arsenic trioxide, retinoids, ligands of the mitochondrial benzodiazepine receptor, and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), a surface death receptor ligand with potent antitumor activity (16–23). Some of these compounds were

Authors' Affiliations: [1]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts; [2]Department of Internal Medicine, University of Genova, Genova, Italy; and Departments of [3]Internal Medicine I, [4]Radiation Oncology, and [5]Hematology, Oncology and Immunology, University of Tubingen, Tubingen, Germany

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Requests for reprints: Alessio Nencioni, Department of Internal Medicine, University of Genova, V.le Benedetto XV 6, 16132 Genova, Italy. Phone: 39-010-353-8990; Fax: 39-010-353-8650; E-mail: A.Nencioni@gmx.net.

proposed to affect Bcl-2 levels and/or phosphorylation state, whereas the mechanism responsible for sensitization by others is not explained.

Proteasome inhibitors represent a novel class of compounds with promising antitumor activity (24, 25). For reasons that are unclear, the cytotoxic effect of these compounds seems to be selective for transformed, as opposite to normal, cells. Besides, proteasome inhibitors show additive effects when combined with other anticancer agents and were reported to reverse radiotherapy/chemotherapy resistance in tumor cell lines. Bortezomib (Velcade), the first proteasome inhibitor to be evaluated in clinical trials, has recently been approved by the Food and Drug Administration for the treatment of relapsed and refractory multiple myeloma (26, 27). Overall, the favorable toxicity profile of these new drugs and the remarkable effects obtained in preclinical and clinical studies have encouraged the evaluation of proteasome inhibitors for the treatment of malignancies of different histology (26–30). In this context, recent observation has revealed promising activity in non-Hodgkin's lymphoma (27, 28).

Whether and to which extent Bcl-2 overexpression affects the cytotoxic activity of proteasome inhibitors is a clinically relevant issue. Following initial reports that proteasome inhibition would overcome Bcl-2 protective function (31, 32), recent evidence indicates that a Bcl-2 excess may actually reduce the antitumor effect of proteasome inhibition (31, 33–35).

In the present study, we have made use of Jurkat lymphoma cells engineered to overexpress Bcl-2 to determine the effect of this molecule on apoptosis induced by proteasome inhibitors, antiblastic drugs, TRAIL, and combinations of these agents.

Materials and Methods

Cells and reagents. The medium used for cell culture was RPMI 1640 for Jurkat cells and DMEM for 293 cells (Life Technologies, Grand Island, NY). Culture medium was supplemented with 10% inactivated FCS, 50 nmol/L 2-mercaptoethanol, and antibiotics (all purchased from Life Technologies). Stable transfectants of Jurkat cells overexpressing Bcl-2 were described previously (22, 36, 37). Benzyloxycarbonil-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Calbiochem (Darmstadt, Germany). MG132, epoxomicin, etoposide, doxorubicin, and 3,3′-dihexyloxacarbocyanine iodide were all obtained from Sigma-Aldrich (St. Louis, MO). Bortezomib was obtained from the pharmacy of S. Martino Hospital (Genova, Italy). Soluble human recombinant TRAIL was by Alexis Biochemicals (San Diego, CA). Recombinant human tumor necrosis factor-α was from PeproTech (Rocky Hill, NJ).

Detection of cell death. For all assays, 5×10^4 cells were seeded in 96-well plates and cultured in the presence of different stimuli in a final volume of 200 μL. Cell viability was determined by staining with 5 μg/mL propidium iodide (PI) and flow cytometry (22). Specific death was calculated as follows: 100 × ([experimental sample (%) − spontaneous sample (%)] / [100% − spontaneous sample (%)]). Spontaneous cell death was required to be <25%.

Immunoblotting. Cell lysates were generated from 1.5×10^6 cells by directly resuspending cell pellets in SDS sample buffer [6.25 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.005% bromophenol blue; Boston Bioproducts, Boston, MA]. Cell lysates were immediately boiled at 100°C for 10 minutes and stored at −20°C for subsequent use. Proteins were separated on a SDS-polyacrylamide gel and electroblotted to a polyvinylidene fluoride membrane (Pall Gelman Laboratory, Ann Arbor, MI). Proteins were visualized by probing the membranes with the following antibodies: anti-caspase-3, anti-caspase-8, anti-caspase-9, anti–poly(ADP-ribose) polymerase (PARP) (Cell Signaling Technology, Beverly, MA), anti-Bcl-2, anti-Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-γ-tubulin (Sigma-Aldrich).

Detection of cytochrome c and SMAC/Diablo release. Lysates were obtained by resuspending 2.5×10^6 cells in 100 μL of 0.025% digitonin (Sigma-Aldrich) in a lysis buffer [250 mmol/L sucrose, 20 mmol/L HEPES (pH 7.4), 5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin]. After 10-minute incubation at 4°C, cells were centrifuged (2 minutes at 13,000 × *g*) and the supernatant (cytosolic fraction) was removed and frozen at −20°C for subsequent use. The cytosolic fraction was separated on a SDS-15% polyacrylamide gel and transferred to a polyvinylidene fluoride membrane as described above. Anti–cytochrome *c* and anti-SMAC/Diablo antibodies both were obtained from Becton Dickinson (Franklin Lakes, NJ).

Flow cytometric assay of mitochondrial transmembrane potential. Cells (2×10^6 per well) were incubated in 0.5 mL culture medium in 24-well plates in the presence of different stimuli. 18 hours later, cells were harvested, incubated for 15 minutes in culture medium containing 40 nmol/L 3,3′-dihexyloxacarbocyanine iodide, and subsequently analyzed by flow cytometry.

Jurkat cell transduction. 293 Cells were used as packaging cells for retrovirus production. 293 Cells (4×10^6) per 4 mL culture medium were transfected in 60 mm round culture dishes using calcium phosphate method with 3 μg VSVG, 3 μg gag/pol, and 6 μg MIG or MIG expressing a dominant-negative mutant of IκBα (IκBαM; a gift of Dr. Inder M. Verma, The Salk Institute for Biological Studies, La Jolla, CA; refs. 38, 39). Jurkat cell infection with viral supernatant was done twice, 48 and 72 hours after transfection of 293 cells. Viral supernatant (2 mL) was added to 2×10^5 Jurkat cells per well in 24-well plates in the presence of polybrene and HEPES buffer. Spin infection was done at 1,000 × *g* for 90 minutes at 37°C. Subsequently, supernatants were removed and replaced with prewarmed RPMI 1640–based medium. Efficiency of infection (as detected by enhanced green fluorescent protein expression) and cell viability were determined by PI cell staining (see above) and flow cytometry.

Results

Efficacy of antiblastic agents, TRAIL, and proteasome inhibitors and combinations of these for the induction of apoptosis in Bcl-2-overexpressing Jurkat cells. The effect of Bcl-2 overexpression on proteasome inhibitor–induced apoptosis was evaluated in the Jurkat cell model. The compounds used to this purpose included bortezomib and MG132, two potent and reversible proteasome inhibitors, and the epoxyketone epoxomicin, which blocks the proteasome in an irreversible fashion (40). Here, we found that, similar to what was observed for the anticancer drugs etoposide and doxorubicin, cell death in response to proteasome inhibitors was almost completely blocked by an excess of Bcl-2 (Fig. 1A), which is consistent with a primary involvement of the intrinsic mitochondrial pathway in proteasome inhibitor–induced apoptosis. In the case of TRAIL, Bcl-2 overexpression prevented apoptosis induction to a lesser extent, thus indicating that blockade of the intrinsic apoptotic pathway only partially antagonizes the apoptotic cascade triggered by the death receptor ligand (Fig. 1B).

Given the observed resistance to apoptotic stimuli that is associated with Bcl-2 up-regulation, we tested the effects of combining proteasome inhibitors with antiblastic drugs or proteasome inhibitors with TRAIL in Bcl-2-overexpressing cells.

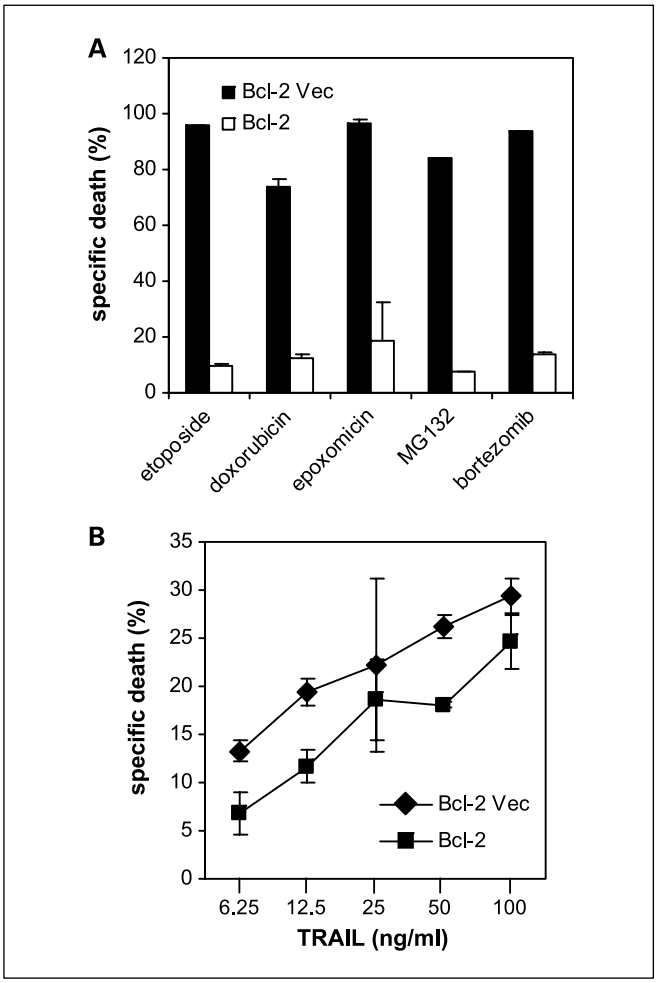

Fig. 1. Effect of Bcl-2 overexpression on apoptosis induced by proteasome inhibitors, anticancer drugs, and TRAIL. *A*, Jurkat cells engineered to overexpress Bcl-2 and the respective vector control cells were exposed for 36 hours to etoposide (25 μg/mL), doxorubicin (0.8 μmol/L), epoxomicin (0.4 μmol/L), MG132 (0.5 μmol/L), or bortezomib (50 ng/mL). Thereafter, cells were harvested and dead cells were quantified by flow cytometry after staining with PI. *Columns*, means of triplicates; *bars*, SD. *B*, Bcl-2-overexpressing and vector control Jurkat cells were incubated for 36 hours with TRAIL at the indicated concentrations. Subsequently, cell death was quantified by flow cytometric analysis of PI-stained cells. *Points*, means of triplicates; *bars*, SD.

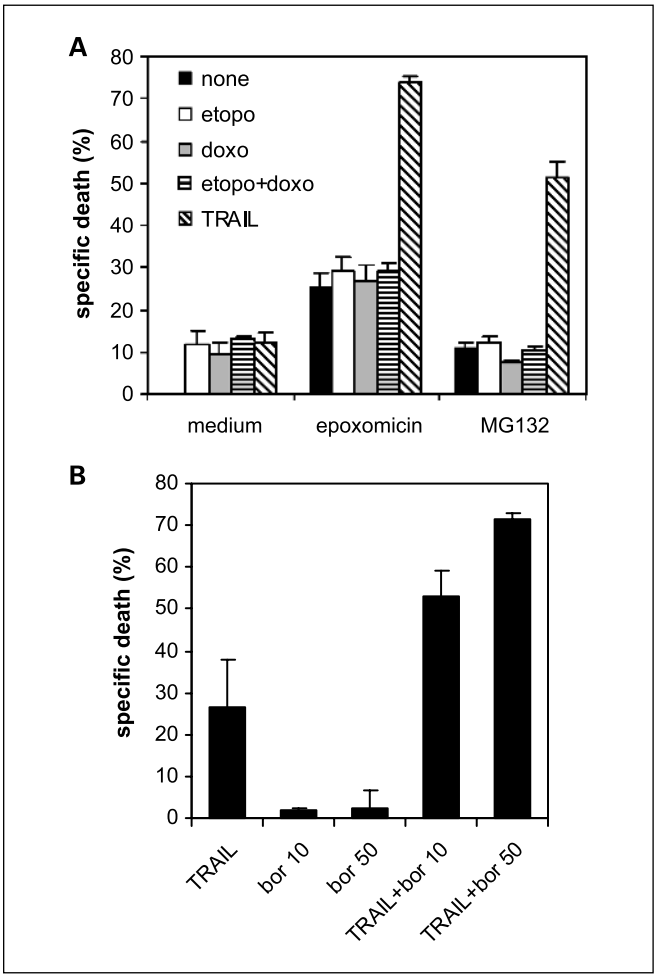

Fig. 2. Proteasome inhibitors cooperate with TRAIL but not with antiblastic drugs to induce apoptosis in Bcl-2-overexpressing cells. *A*, Bcl-2 Jurkat cells were treated for 36 hours with etoposide (25 μg/mL; *etopo*), doxorubicin (0.8 μmol/L; *doxo*), TRAIL (25 ng/mL), epoxomicin (0.4 μmol/L), MG132 (0.5 μmol/L), or combinations of these stimuli as indicated. Thereafter, PI-positive cells were quantified by flow cytometry. *Columns*, means of triplicates; *bars*, SD. *B*, Bcl-2 Jurkat cells were treated for 36 hours with 25 ng/mL TRAIL, 10 or 50 ng/mL bortezomib (*bor*), or combined bortezomib and TRAIL. Cell death was determined by PI cell staining and flow cytometry. *Columns*, means of triplicates; *bars*, SD.

The combination of anticancer drugs (etoposide, doxorubicin, and oxaliplatin) and TRAIL was evaluated previously and it was found to synergistically kill Bcl-2- and Bcl-x_L-overexpressing lymphoma cells (22). Here, we observed that adding proteasome inhibitors to etoposide, doxorubicin, or the combination of the two drugs resulted in a less than additive proapoptotic effect in the presence of an excess of Bcl-2 (Fig. 2A). Conversely, proteasome inhibitors efficiently killed Bcl-2-overexpressing cells when added to TRAIL (Fig. 2A). The cooperative enhancement of TRAIL cytotoxicity by proteasome inhibitors was observed for TRAIL concentrations as low as 6 ng/mL (data not shown). Importantly, clinically achievable bortezomib concentrations also reproduced this effect (Fig. 2B; ref. 29).

Proteasome inhibitors plus TRAIL produce mitochondria dysfunction in the presence of overexpressed Bcl-2. Bcl-2 works by preventing release of proapoptotic factors from the mitochondrial intermembrane space in response to apoptotic stimuli (1). Hence, we monitored the levels of cytosolic cytochrome *c* and SMAC/Diablo in wild-type and Bcl-2-overexpressing Jurkat cells on stimulation with the proteasome inhibitor epoxomicin, etoposide, TRAIL, combined proteasome inhibitor and etoposide, or combined proteasome inhibitor and TRAIL. Addition of any of the apoptotic stimuli or combinations of them produced cytosolic relocalization of cytochrome *c* and SMAC/Diablo in the wild-type cells (Fig. 3A). Conversely, on Bcl-2 overexpression, proteasome inhibitor plus TRAIL was the only stimulation condition that was able to determine cytochrome *c* and SMAC/Diablo release to comparable levels with the wild-type control (Fig. 3A). The observed leak of mitochondrial proapoptotic factors in response to the combination of TRAIL with proteasome inhibitor was prevented by the broad-spectrum caspase inhibitor zVAD-fmk in Bcl-2-overexpressing cells but not in the corresponding control cells. With this respect, we have observed previously that, in the absence of Bcl-2 overexpression, proteasome inhibitor–induced cytochrome *c* release occurs upstream of caspases, whereas

cytochrome *c* freeing in response to TRAIL is blocked by caspases inhibition (41). This is in line with current knowledge indicating that surface death receptor ligands can also trigger the intrinsic death pathway, where this effect is largely dependent on caspase-8 (and possibly caspase-3)–mediated Bid cleavage (42). Thus, these data confirm that proteasome inhibitors suffice at determining mitochondrial proteins freeing independent of caspases in wild-type cells, whereas caspase activity, which is likely contributed by TRAIL stimulation (see below), is required to produce opening of the mitochondrial membrane in the presence of overexpressed Bcl-2.

Loss of mitochondrial transmembrane potential is another indicator of mitochondria dysfunction. Consistent with the above-reported observation of cytosolic release of mitochondrial proteins, we found that a Bcl-2 excess impeded mitochondria depolarization in response to proteasome inhibitor and TRAIL when these were taken alone (Fig. 3B). However, combining them readily promoted mitochondrial transmembrane potential dissipation. Altogether, these observations indicate that the combination of proteasome inhibitor and TRAIL is sufficient at overcoming mitochondria resistance that is determined by Bcl-2 overexpression.

Combined TRAIL and proteasome inhibitor amplify the apoptotic cascade in Bcl-2-overexpressing cells. Caspase activation accompanies and mediates cell demise in response to several cytotoxic stimuli. In wild-type Jurkat cells, treatment with etoposide, TRAIL, proteasome inhibitor, or their combinations resulted in activation (as detected by cleavage) of caspase-3, -8, and -9 and cleavage of PARP, a well-described caspase target (Fig. 4). In Bcl-2-overexpressing Jurkat cells, TRAIL produced some cleavage of caspase-8 and -3 and poly(ADP-ribose) polymerase (Fig. 4). Addition of epoxomicin to TRAIL strongly amplified this effect leading to enhanced activation of casapase-3, -8, and -9 and PARP cleavage. zVAD-fmk blocked the caspase cascade in both control and Bcl-2 cells. It is of note that, when Bcl-2 is overexpressed, caspase-3 cleavage products in response to TRAIL are mostly represented by p20, which was reported to possess reduced activity (43). On the contrary, proteasome inhibitor plus TRAIL determined the appearance of the active caspase-3 fragment p17.

Effect of proteasome inhibition and TRAIL on Bcl-2 and Mcl-1. The above-reported data show that the combination of proteasome inhibitors and TRAIL produces mitochondria disassembling irrespective of overexpressed Bcl-2. Therefore, we monitored the effect of these treatment conditions on Bcl-2 protein. Addition of proteasome inhibitor to TRAIL, although not obviously affecting Bcl-2 levels, was found to induce caspase-mediated Bcl-2 cleavage (Fig. 5). The cleaved Bcl-2 form appeared only in the presence of Bcl-2 overexpression while remaining almost undetectable in wild-type cells. The reason for this effect is unclear but may be related to the high Bcl-2 levels in the overexpressing cells, which make it easier to detect the cleaved fragment. Because Bcl-2

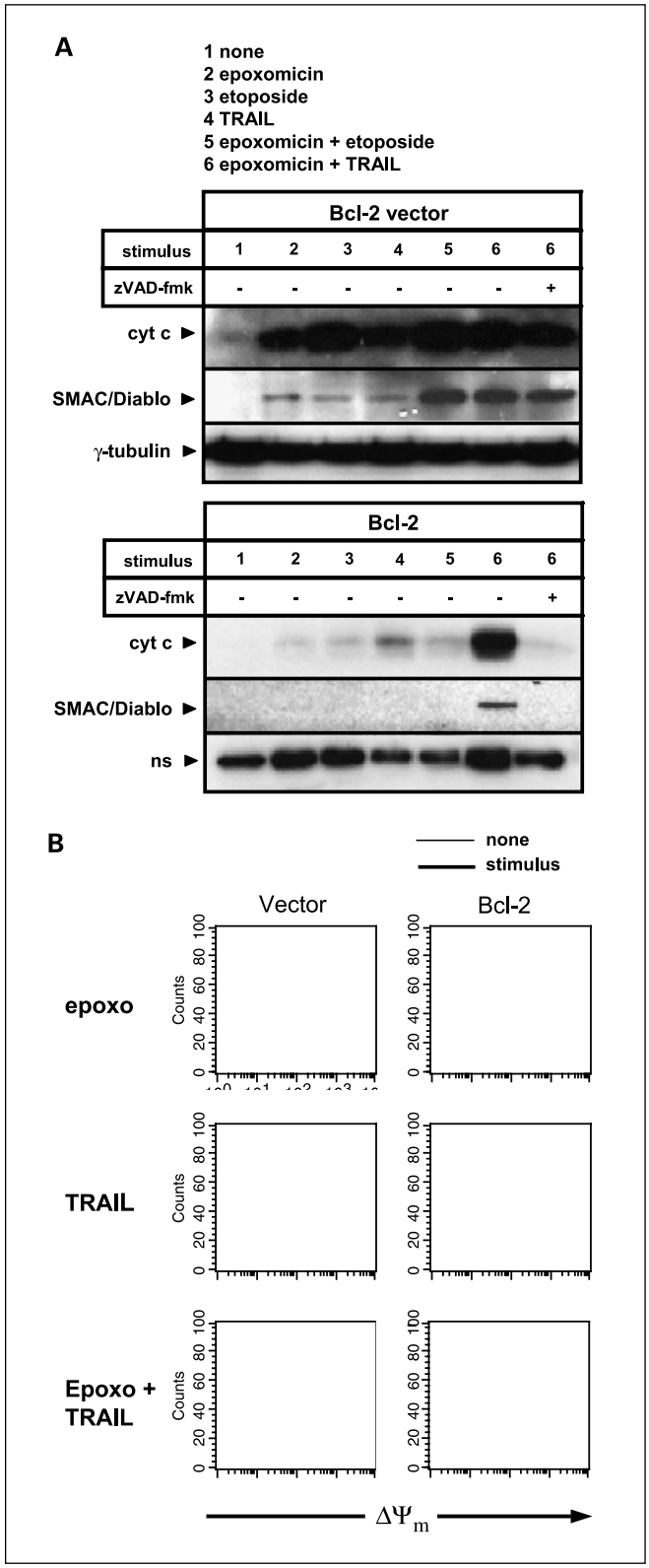

Fig. 3. Combined TRAIL and proteasome inhibitor produce mitochondrial dysfunction irrespective of Bcl-2 overexpression. *A*, Bcl-2-overexpressing and vector control Jurkat cells were preincubated for 1 hour with regular medium or 100 μmol/L zVAD-fmk. Thereafter, cells were treated with or without epoxomicin (0.4 μmol/L), etoposide (25 μg/mL), TRAIL (25 ng/mL), or combinations of these stimuli. Eight hours later, the cytosolic fraction was isolated and cytochrome *c* (*cyt c*) and SMAC/Diablo levels were determined by immunoblotting. γ-Tubulin or a nonspecific (*ns*) cross-reactive immune band was used as equal protein loading control. *B*, Bcl-2-overexpressing and control Jurkat were treated for 18 hours with 0.4 μmol/L epoxomicin (*epoxo*), 25 ng/mL TRAIL, or combined epoxomicin and TRAIL. Subsequently, cells were harvested, stained with 3,3′-dihexyloxacarbocyanine iodide, and analyzed by flow cytometry.

cleavage converts this molecule to a bax-like death effector, which further activates downstream caspases (44), the observed effect could play a role in the cytotoxic synergy between proteasome inhibitors and TRAIL in the presence of overexpressed Bcl-2.

Importantly, proteasome inhibition causes accumulation of Mcl-1, another antiapoptotic Bcl-2 family member that is highly dependent on proteasome activity for its degradation (1, 45–47). Mcl-1 plays a key role in survival of B-cell lymphoma and myeloma cells (46, 48–50) and we have recently obtained evidence that this molecule is also required to prevent cell death in Jurkat cells (41). Besides, Mcl-1 elimination was suggested to be necessary for the initiation of apoptosis in response to cytotoxic stimuli (45). Thus, these observations raise the possibility that Mcl-1 accumulation via proteasome inhibitors may limit the lethality of these compounds. Here, we found that proteasome inhibition leads to increased Mcl-1 levels in both wild-type and Bcl-2-overexpressing Jurkat cells (Fig. 5). This effect is partially counteracted in wild-type cells by the concomitant Mcl-1 degradation mediated by caspases (Fig. 5; data not shown). Consistent with this, in the presence of up-regulated Bcl-2, which blocks the apoptotic cascade, Mcl-1 accumulation is more pronounced. However, addition of TRAIL (but not of etoposide) to the proteasome inhibitor cancels Mcl-1 expression, this effect being inhibited by zVAD-fmk.

Nuclear factor-κB inhibition does not sensitize Bcl-2-overexpressing cells to TRAIL. Proteasome inhibition reduces nuclear factor-κB (NF-κB) signaling by preventing degradation of the NF-κB inhibitor IκBα and this effect has been proposed to account, at least in part, for the antitumor activity of proteasome inhibitors (51). This is because NF-κB activity is increased in many types of human cancer and is responsible for the expression of apoptosis inhibitors, such as cIAP1/2 and XIAP (52). To evaluate whether NF-κB inhibition would play a role in the observed cooperation of proteasome inhibitors and TRAIL in Bcl-2-overexpressing cells, we transduced wild-type and Bcl-2-overexpressing Jurkat with a dominant negative form of IκBα (IκBαM). Although not sufficient at inducing cell death, IκBαM expression predisposed wild-type cells to apoptosis in response to tumor necrosis factor-α (Fig. 6A and B). This is consistent with previous reports (39) and confirms the effectiveness of NF-κB disabling by means of this genetic approach. However, IκBαM failed to enhance susceptibility of Bcl-2-overexpressing Jurkat cells to TRAIL (as well as to anticancer drugs; Fig. 6C), suggesting that NF-κB silencing via proteasome inhibitors is unlikely to explain the enhancement of TRAIL activity in the presence of up-regulated Bcl-2.

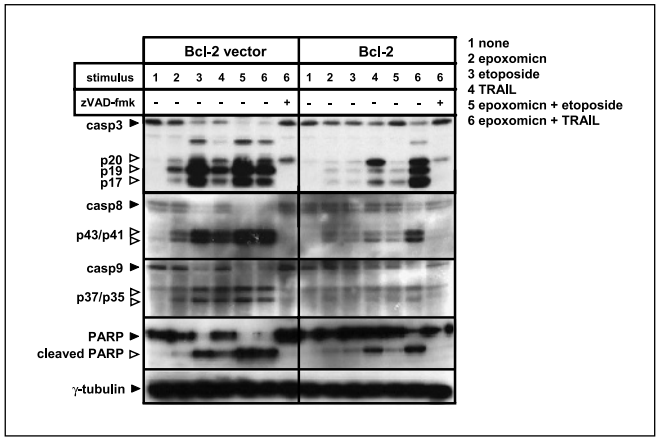

Fig. 4. TRAIL plus proteasome inhibitor amplify the apoptotic cascade in Jurkat cells with up-regulated Bcl-2. Following a 1-hour pretreatment with zVAD-fmk (100 μmol/L), Bcl-2-overexpressing and vector control Jurkat cells were incubated in the presence or absence of epoxomicin (0.4 μmol/L), etoposide (25 μg/mL), TRAIL (25 ng/mL), or combinations of these stimuli. Subsequently, cells were harvested and used for cell lysates preparation. Caspase-3 (*casp3*), caspase-8 (*casp8*), and caspase-9 (*casp9*) and poly(ADP-ribose) polymerase (*PARP*) cleavage were determined by Western blotting. Equal protein loading was assessed by reprobing the membranes with an anti-γ-tubulin antibody.

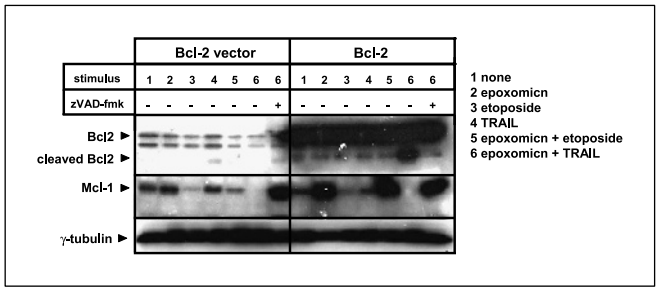

Fig. 5. Effect of combined proteasome inhibitor and TRAIL on Bcl-2 and Mcl-1. Bcl-2-overexpressing and vector control Jurkat cells were incubated for 1 hour with zVAD-fmk (100 μmol/L) and subsequently treated with or without epoxomicin (0.4 μmol/L), etoposide (25 μg/mL), TRAIL (25 ng/mL), or combinations of these stimuli. Subsequently, cell lysates were prepared and Bcl-2 and Mcl-1 were detected by immunoblotting.

Discussion

In the present study, we found that the cytotoxic activity of proteasome inhibitors is strongly inhibited by overexpression of the antiapoptotic oncogene Bcl-2, which prevents activation of the mitochondrial pathway and the downstream apoptotic cascade in response to these drugs. However, resistance to proteasome inhibitors mediated by Bcl-2 is removed by addition of TRAIL. This combination produces mitochondria disassembling irrespective of Bcl-2 up-regulation and results in efficient apoptosis induction.

Our finding that Bcl-2 prevents proteasome inhibitor–induced apoptosis is supported by recent reports indicating the involvement of the intrinsic apoptotic pathway in the cytotoxic effect of these compounds (53–55). The discrepancy with the results reported by other groups that failed to detect inhibition of proteasome inhibitor lethality by Bcl-2 up-regulation (32) can be tentatively explained based on the Bcl-2 expression levels used in the different experimental systems. Actually, Bcl-2 expression methods that lead to lower protein levels compared with the cell clones that we used in this study only determine a weak inhibition of apoptosis in response to cytotoxic stimuli.[6] On the other hand, the mechanism that induces cytochrome *c* and SMAC/Diablo release in response to proteasome inhibition is unknown, although a deregulated unfolded protein response with endoplasmic reticulum stress and/or oxidative stress have both

[6] Unpublished observations.

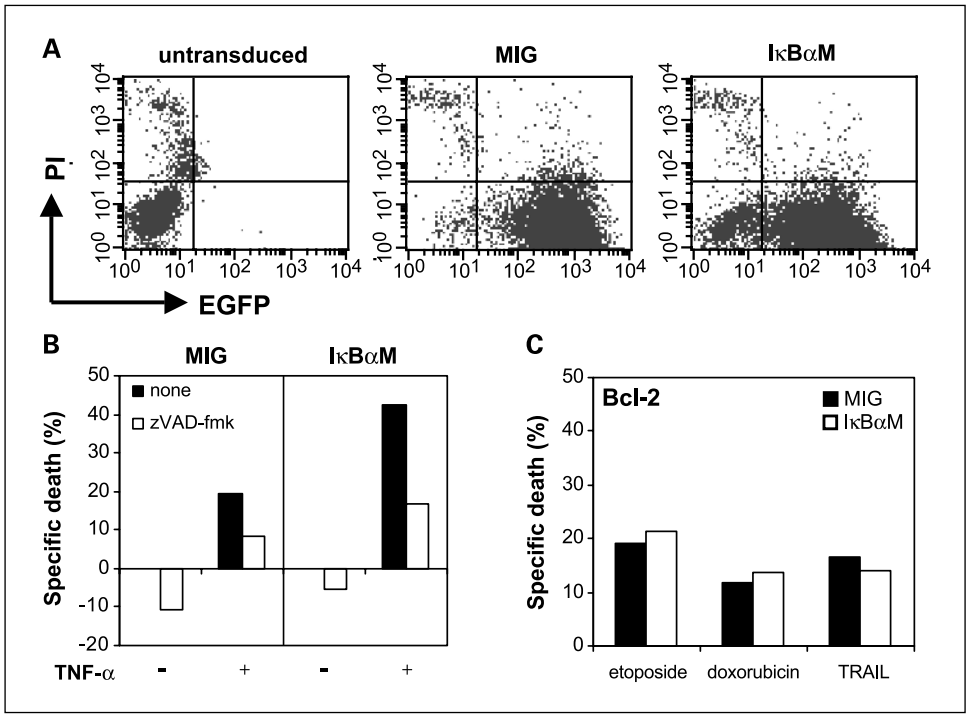

Fig. 6. A dominant negative form of IκBα fails to enhance TRAIL cytotoxicity in the presence of overexpressed Bcl-2. *A*, Jurkat cells were transduced with IκBαM or the respective vector (MIG). Forty-eight hours following transduction, cells were harvested, stained with PI, and analyzed by flow cytometry. *B*, Following a 1-hour incubation with 100 μmol/L zVAD-fmk, Jurkat cells that were transduced previously with IκBαM or the corresponding vector MIG were treated with or without 100 ng/mL recombinant human tumor necrosis factor-α (*TNF-α*). Twenty-four hours later, cells were harvested and dead cells were determined by flow cytometry after staining with PI. *Columns*, means of duplicates. *C*, Bcl-2–overexpressing Jurkat cells were transduced with IκBαM or MIG vector. The obtained cell lines were treated for 36 hours with 25 μg/mL etoposide, 0.8 μmol/L doxorubicin, or 25 ng/mL TRAIL. Subsequently, cell death was assessed by PI staining and flow cytometry. *Columns*, means of duplicates.

been proposed as triggers of the mitochondrial apoptotic machinery (54, 56).

Proteasome inhibitors were reported previously to cooperate with anticancer drugs for the induction of apoptosis in chemoresistant cells (1, 2). However, here, we found that combining etoposide or doxorubicin (or both the anticancer drugs) with proteasome inhibitors fails to determine significant cytotoxicity when Bcl-2 is overexpressed. Thus, combinations of apoptotic stimuli, which all converge on the same (intrinsic) apoptotic pathway, may not be productive in the presence of Bcl-2 up-regulation.

On the contrary, addition of TRAIL to proteasome inhibitors resulted in a highly synergistic proapoptotic effect in Bcl-2–overexpressing cells. Importantly, although not sufficient to promote cytochrome *c* and SMAC/Diablo release when Bcl-2 is up-regulated, TRAIL achieves this effect in the presence of proteasome inhibitors. Thus, combining these two stimuli overcomes the blockade of the mitochondrial pathway imposed by Bcl-2. This is associated with amplification of the caspase cascade and apoptosis progression.

A cooperative interaction between proteasome inhibitors and TRAIL has recently been reported for cells that exhibit other abnormalities in the apoptotic cascade, including XIAP expression, bax and caspase-9 deficiency, and increased Bcl-x_L levels (43, 57, 58). The mechanism by which combined TRAIL and proteasomal inhibitors allow mitochondria dysfunction seems to require bak, a proapoptotic Bcl-2 family member that releases mitochondrial intermembrane space proteins, including cytochrome *c* and SMAC/Diablo (57). Yet, this effect remains largely elusive. Here, we found that concomitant exposure of cells with up-regulated Bcl-2 to TRAIL and proteasome inhibitors determines Bcl-2 cleavage and production of a Bcl-2 fragment with proapoptotic function (44). Besides, the combination of these two stimuli induces Mcl-1 elimination, which accelerates cell death in Jurkat cells (41). Both of these effects are likely to contribute to the cooperation between proteasome inhibitors and TRAIL at the mitochondrion level. However, because Bcl-2 fragmentation and Mcl-1 degradation are likely mediated by caspases (41, 44, 59–61), we cannot exclude that these events actually intervene as downstream amplification mechanisms instead of constituting the causative factor that triggers the mitochondrial pathway.

NF-κB inhibition has been reported to mediate the antitumor activity of proteasome inhibitors (51). However, recent evidence indicates that this effect is unlikely to be the sole responsible for apoptosis in response to these compounds (1, 2). Here, we found that disabling NF-κB signaling by a dominant negative mutant of IκBα does not sensitize Bcl-2–overexpressing lymphoma cells to TRAIL (or anticancer drugs). Besides, peptide NF-κB inhibitors also failed to act in a proapoptotic fashion in our cellular system.[7] This is consistent with the recent report showing that proteasome inhibitors remove resistance to TRAIL independent of NF-κB in primary keratinocytes (43). Therefore, we suggest that the cooperative cytotoxicity of TRAIL and proteasome inhibitors in chemoresistant cells is probably unrelated to inhibition of NF-κB.

In conclusion, this study indicates that administration of proteasome inhibitors together with TRAIL efficiently induces apoptosis in chemoresistant Bcl-2–overexpressing lymphoma cells. Whether and how this effect will translate into clinical applications needs careful evaluation based on preliminary observations that normal tissues may also be sensitized to TRAIL by proteasome inhibition (43).

[7] Unpublished observations.

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