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ARTICLE

Proteasome Inhibitors Sensitize Colon Carcinoma Cells to TRAIL-Induced Apoptosis via Enhanced Release of Smac/DIABLO from the Mitochondria

Katalin NAGY,[1,3] Kinga SZÉKELY-SZÜTS,[5] Kamel IZERADJENE,[5] Leslie DOUGLAS,[5] Mike TILLMAN,[5] Helga BARTI-JUHÁSZ,[1] Massimo DOMINICI,[4] Carlotta SPANO,[4] Gian LUCA CERVO,[4] Pierfranco CONTE,[4] Janet A HOUGHTON,[5] Rudolf MIHALIK,[2] László KOPPER,[1,2,3] István PETÁK,[1,3,*]

[1]I[st] Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, [2]Molecular Pathology Research Group, Joint Research Organization of the Hungarian Academy of Science and Semmelweis University, Budapest, [3]Szentágothai János Knowledge Center, Budapest, Hungary; [4]Department of Oncology and Hematology, University of Modena and Reggio Emilia, Modena, Italy; [5]Division of Molecular Therapeutics, Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, TN, USA

The synergistic interaction between proteasome inhibitors and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising approach to induce cell death in tumor cells. However, the molecular and biochemical mechanisms of this synergism have been proven to be cell type specific. We therefore focused our investigation on TRAIL-resistant colon carcinoma cells in this study. DNA fragmentation, mitochondrial membrane depolarization and increased caspase-3-like enzyme activity was exclusively induced only by combined treatment with proteasome inhibitors (epoxomicin, MG132, bortezomib/PS-341) and TRAIL. The expression level of anti-apoptotic proteins (XIAP, survivin, Bcl-2, Bcl-X_L), regulated by NF-κB transcription factor, was not effected by any of these treatments. TRAIL alone induced only partial activation of caspase-3 (p20), while the combination of TRAIL and proteasome inhibition led to the full proteolytic activation of caspase-3 (p17). Only the combination treatment induced marked membrane depolarization and the release of cytochrome c, HtrA2/Omi and Smac/DIABLO. Apoptosis-inducing factor (AIF) was not released in any of these conditions. These results are consistent with a model where the full activation of caspase-3 by caspase-8 is dependent on the release of Smac/DIABLO in response to the combined treatment. This molecular mechanism, independent of the inhibition NF-kB activity, may provide rationale for the combination treatment of colon carcinomas with proteasome inhibitors and recombinant TRAIL or agonistic antibody of TRAIL receptors. (Pathology Oncology Research Vol 12, No 3, 133–142)

Key words: TRAIL, epoxomicin, MG132, bortezomib/PS-341, Smac/DIABLO, colon carcinoma

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Correspondence: István PETÁK, MD, PhD, 1[st] Department of Pathology and Experimental Cancer Research, Semmelweis University, Üllői út 26., Budapest, H-1085, Hungary. Tel/Fax: (36) (1) 4591500, ext: 4453, E-mail: petak@kkk.org.hu

Abbreviations: Ac-DEVD-AMC: Ac-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin; z-VAD-fmk: benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone, z-IETD-fmk: benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone, MG132: benzyloxycarbonyl-Leu-Leu-Leu-aldehyde, AIF: apoptosis-inducing factor; IAP: inhibitor of apoptosis protein; XIAP: X-linked IAP, Epo: epoxomicin

Introduction

Proteasome inhibitors represent novel anti-cancer drugs which interact with the proteasome-ubiquitin pathway. Bortezomib/PS-341 (Velcade) was the first proteasome inhibitor approved by the Food and Drug Administration for clinical use.[1] The 26S proteasome is a protease complex with three distinct catalytic activities. It is responsible for intracellular protein turnover in eukaryotic cells, therefore it plays a crucial role in regulation of various cellular functions.[2-5] The role of the proteasome and its inhi-

bition in the regulation of apoptosis is an intensively studied area since most of the pro- and anti-apoptotic proteins are identified as proteasome substrates.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) acts through the pro-apoptotic TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors in tumor cells without harming normal cells, and is currently being tested in phase I clinical trial as a novel anti-cancer agent.[6-8] However, not all human cancer cell lines are sensitive to TRAIL due to intrinsic or acquired TRAIL resistance.[9]

Previous studies have shown that activation of NF-κB can inhibit apoptosis induced by a number of stimuli; it is also known that TRAIL can activate NF-κB through the death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5, and decoy receptor TRAIL-R4/DcR2, resulting in the upregulation of anti-apoptotic proteins including Bcl-2, XIAP, IAP1/2 and c-FLIP.[10-13]

Proteasome inhibitors can interrupt the activation of NF-κB, which is considered to be their main mechanism of anti-tumor action in certain tumor types, in particular myelomas. Furthermore, inhibition of NF-κB signaling can lead to significant chemosensitization and synergistic effects with cytotoxic agents including TRAIL.[14-16]

Mechanisms for synergistic interaction between TRAIL and proteasome inhibitors independent of NF-κB signaling have also been suggested. These include upregulation of the cell surface TRAIL receptors and the subsequent increased release of pro-apoptotic mitochondrial proteins via a BAK-dependent mechanism.[17] Most recently two papers have reported upregulation of the BH3-only protein BIK by proteasome inhibitors.[18,19] BIK was implicated in both the direct cytotoxic activity of proteasome inhibitors[19] and also in the increased sensitivity to TRAIL-induced apoptosis via activating the mitochondrial pathway.[20]

Our results indicate that although the proteasome inhibitor epoxomicin upregulates the TRAIL-R2/DR5 receptor, colon carcinoma cells express sufficient amounts of this receptor to partially activate caspase-3 in response to TRAIL/APO2L treatment alone without releasing any pro-apoptotic mitochondrial proteins or inducing DNA fragmentation. On the other hand, epoxomicin alone induced the release of cytochrome c without subsequent activation of caspase-3 or DNA fragmentation. However, the combined treatment with TRAIL/APO2L and epoxomicin released Smac/DIABLO, which was accompanied by the full proteolytic activation of caspase-3 and extensive apoptotic DNA fragmentation. The current model indicates that not only can proteasome inhibitors restore sensitivity of tumor cells to TRAIL/APO2L, but activation of the death-inducing signaling complex (DISC) can also overcome resistance to proteasome inhibitors caused by non-functional apoptosomes downstream of the mitochondria.

These results indicate that the mechanism of molecular interaction between proteasome inhibitors and TRAIL is tumor type specific. Our study provides the first detailed description of synergistic effect of these treatments in human colon carcinoma cells.

Materials and methods

Cell culture

The RKO colon carcinoma cell line was obtained from ATCC and was maintained in RPMI-1640 medium (BioWhittaker) supplemented with 10% FCS (BioWhittaker) and L-glutamine (Sigma) at 37°C, in an atmosphere of 95% air and 5% CO_2. For all experiments cells growing in the exponential phase were used. Plastic materials were purchased from Sarstedt.

Apoptosis assays

Cells were plated at a density of 10^5 cells/ml in 24-well plates. After overnight attachment cells were incubated in the presence or absence of various treatments. Stock solutions of all compounds except for TRAIL were made in DMSO (dimethylsulfoxide, from Sigma) at concentrations that the final dilutions contained less than 0.1% DMSO. Recombinant TRAIL (aa. 114-281) was produced as described previously.[21] During time course experiments RKO cells were treated with TRAIL for up to 13 h in the presence or absence of pretreatment (30 min) with various proteasome inhibitors [epoxomicin 1 μM, (Boston Biochemicals); MG132 3 μM (Calbiochem) and bortezomib/PS-341 3 μM (generous gift from Millennieum Inc., Cambridge, MA, USA)]. Control samples were exposed to either DMSO (as vehicle) or inhibitors only. In separate experiments, RKO cells were plated and pretreated for 30 min with 10 μM z-IETD-fmk (Calbiochem) or 50 μM z-VAD-fmk (Calbiochem), then incubated for further 30 min in the presence or absence of the proteasome inhibitor epoxomicin (1 μM). Following pretreatments with the inhibitors cells were incubated for 5 or 9 h in the presence or absence of TRAIL (60 ng/ml). At the end of incubation times, cells were harvested (both floating and attached) and fixed in 70% ethanol (-20°C) for 30 min, and stored at -20°C until analysis. Fragmented DNA was extracted by using alkaline buffer (200 mM disodiumphosphate, pH 7.8, adjusted with 200 mM Na-citrate) supplemented with 100 μg/ml DNase-free RNaseA (Sigma). Samples were left at RT for 20 min followed by the addition of 5 ml ethidium bromide (final concentration 10 μg/ml). After a further 10 min, samples were measured with a flow cytometer (FACScan, Becton Dickinson) and cell cycle and proportion of sub-G1 (apoptotic) cells were calculated. Gating was evaluated as previously described.[21]

Caspase activity assays

RKO cells were incubated for 5 h in the presence or absence of epoxomicin (1 μM) and/or TRAIL (60 ng/ml), and subsequently washed twice in PBS then resuspended in 100 ml of caspase activity puffer (CAP: 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 0.2% CHAPS [pH 7.3]) supplemented with 10 mM dithiothreitol (DTT). Caspase activity was detected with Ac-DEVD-AMC (25 μM final concentration) added to the cell suspension in a further 100 ml CAP containing 1% Triton X-100 (Sigma). Lysates were incubated for 30 min at 37°C then fluorescence was detected by a fluorescence plate reader (Fluoroskan Ascent FL, Labsystems) at 380 nm excitation and 460 nm emission wavelength for 20 min. All activity was linear in this time range. Activity was calculated from the rise of activity curves and presented as relative caspase activity compared to control.

Detection of mitochondrial membrane depolarization

RKO cells were plated at a density of 10^5 cells/ml in 24-well plates and treated as described in the caspase activity assay above. 10 nM $DiOC_6$ was added to unfixed cells and incubated for 15 min at 37°C, 5% CO_2. The positively charged $DiOC_6$ accumulates in the intact mitochondria and mitochondria with depolarized membrane accumulate less $DiOC_6$. 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.

Western blot analysis

A T75 culture flask with RKO cells of 70% confluence (about $2x10^7$ cells/flask) was treated with epoxomicin and TRAIL as described above for 3 and 5 h. At the end of incubation times, cells were washed twice in PBS and centrifuged (300 g, 5 min). The pellet was resuspended in 250 μl lysis buffer [3 mM TRIS, 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 1 mM PMSF and protease inhibitor cocktail (Sigma)]. Each sample was boiled for 3 min, and the protein content was determined by the Bradford method. Total protein (10-20 μg) was electrophorated in a

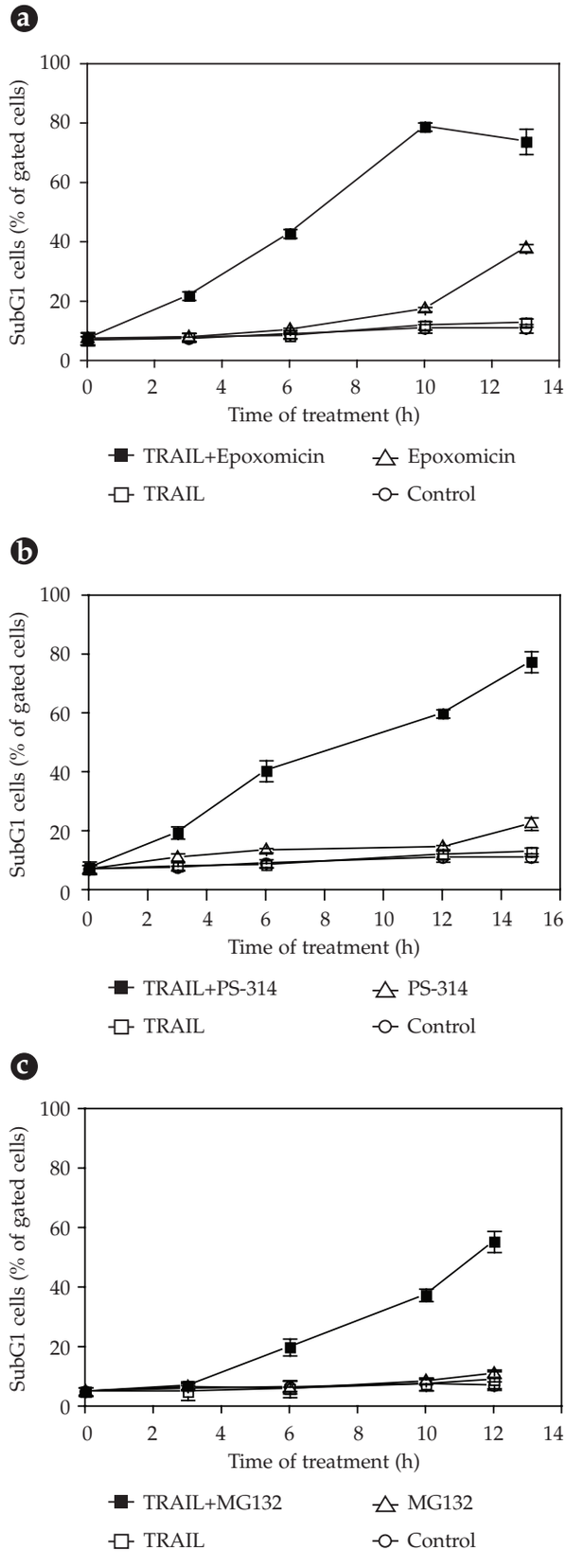


Figure 1. *Proteasome inhibitors sensitize TRAIL-resistant RKO colon carcinoma cells to TRAIL-induced cell death. Cells were left untreated or pre-treated with* ***(a)*** *epoxomicin (Epo; 1 μM),* ***(b)*** *bortezomib/PS-341 (3 μM), or (c) MG132 (3 μM) for 30 min and subsequently treated with TRAIL (60 ng/ml) for increasing incubation times. Fragmented DNA of apoptotic cells was extracted in alkaline buffer and percentage of cells with decreased DNA content (sub-G1) was determined by flow cytometry after ethidium bromide staining. Values indicate the mean ± SD of three determinations.*

4-20% polyacrylamide gel (ready gels, Bio-Rad). Antibodies were purchased from the following sources: caspase-3, caspase-8 and XIAP, MBL; Bcl-2, Bcl-X_L, Translab; survivin, R&D Systems; actin, Sigma. Detection (including secondary antibodies) was performed by using the Vectastain ABC Kit (Vector Laboratories) with ECL Plus (Amersham) as the chemiluminescent substrate.

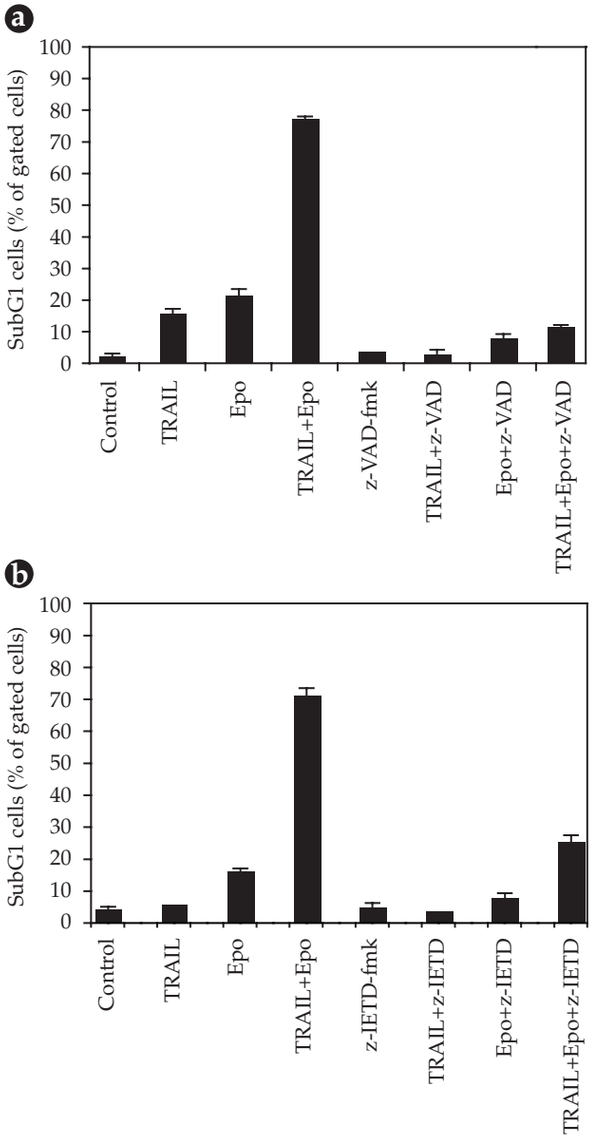


Figure 2. *Apoptotic DNA fragmentation induced by the combined treatment of TRAIL and epoxomicin is dependent on caspase activity. RKO cells were treated with TRAIL (60 ng/ml) and/or epoxomicin (Epo, 1 μM) in the presence or absence of the general caspase inhibitor z-VAD-fmk (50 μM)* ***(a)*** *or the caspase-8 inhibitor z-IETD-fmk (10 μM)* ***(b)*** *and incubated for 9 h. Fragmented DNA of apoptotic cells was extracted and percentage of sub-G1 cells was determined by flow cytometry. Values indicate the mean ± SD of three determinations.*

Examining the release of mitochondrial pro-apoptotic proteins into the cytosol

A T75 culture flask with RKO cells of 80-90% confluence was treated with epoxomicin and TRAIL as described above. At the end of the incubation cells were washed twice in PBS and centrifuged (600 g, 5 min, 4°C). For separation of the cytosolic fraction from the mitochondrial fraction we used ApoAlert Cell Fractionation Kit (Clontech) as described in the user manual. Samples were used for Western blot analysis as described above. Anti-cytochrome c polyclonal antibody was a component of the cell fractionation kit, anti-Smac/DIABLO polyclonal antibody was purchased from MBL, anti-AIF monoclonal antibody from SantaCruz and anti-HtrA2/Omi polyclonal antibody was a kind gift of Dr. Alnemri, ES (Philadelphia, PA, USA). Detection was performed as described above.

Statistical analysis

All experiments were repeated at least three times and results are presented as mean±SD. Statistical analysis was performed by unpaired, two-tailed Student's t-test, and p values <0.05 were considered significant.

Results

Proteasome inhibitors sensitize colon carcinoma cells to TRAIL-induced cell death

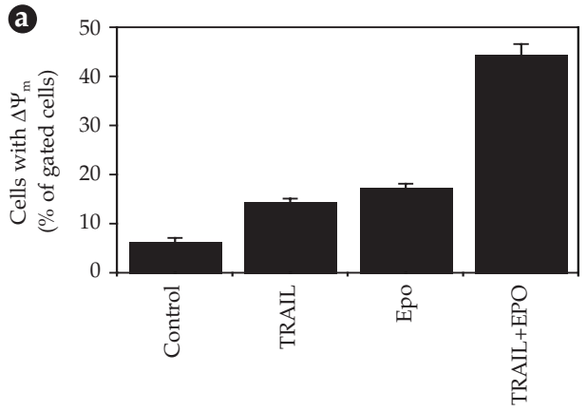
RKO colon cancer cells were pre-incubated in the presence or absence of proteasome inhibitors (epoxomicin: 1 μM; MG132: 3 μM; PS-341: 3 μM) for 30 min and then TRAIL (60 ng/ml) was added for further incubation up to 13 h (*Figure 1*). Cells with apoptotic DNA fragmentation were detected as a sub-G1 population by FACS analysis. TRAIL, MG132 or bortezomib/PS-341 alone did not induce apoptosis. Epoxomicin did not induce apoptosis up to 10 h, but after 13 h the ratio of cells with DNA fragmentation increased to 37%±4%. Combined treatment with TRAIL and epoxomicin induced 40%±3% apoptosis already at 6 h which further increased gradually to 83% at 10 h when it reached a plateau. Similarly, bortezomib/PS-341 in combination with TRAIL induced 41%±5% apoptosis at 6 h and reached 82%±3% at 13 h. The induction of apoptosis by the combined treatment of TRAIL and MG132 was more gradual, but still reaching 63%±5% at 13 h. These results indicate that RKO cells are completely resistant to apoptosis induced by TRAIL or proteasome inhibitors for at least 10 h, while very sensitive to the combined treatment of TRAIL and proteasome inhibitors. Similar results were obtained with HT29 colon carcinoma cells and SKOV3 ovarian carcinoma cells (data not shown).

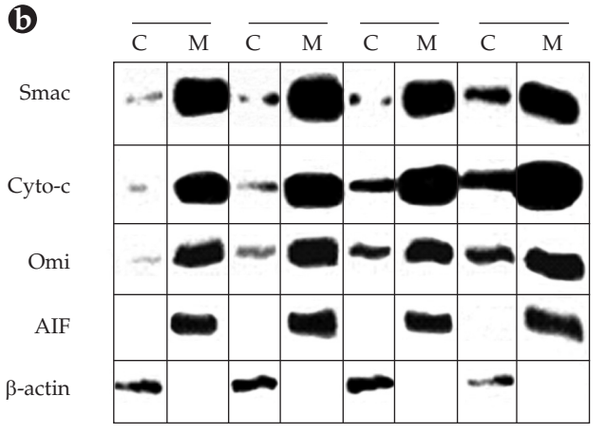
Apoptotic DNA fragmentation induced by TRAIL and proteasome inhibitors depends on caspase activity

z-VAD-fmk is a cell-permeable general inhibitor of caspases, while z-IETD-fmk is a less selective inhibitor of caspase-8, especially at relatively low concentrations (up to 10 μM). Following 30 min pre-incubation with 50 μM z-VAD-fmk or 10 μM z-IETD-fmk and another 30-min pre-treatment with 1 μM epoxomicin, RKO cells were incubated in the presence of 60 ng/ml TRAIL for 9 h. Apoptotic DNA fragmentation was detected by FACS analysis. After combination treatment 78% apoptotic DNA fragmentation was observed, which was completely inhibited by z-VAD-fmk (*Figure 2a*) ($p<0.0001$) and was significantly inhibited by caspase-8 inhibitor z-IETD-fmk ($p<0.01$) (*Figure 2b*). However, the percent of sub-G1 cells remained significantly higher than the control values ($p<0.001$).

Co-treatment of RKO cells with TRAIL and epoxomicin results in mitochondrial membrane depolarization and enhanced release of Smac/DIABLO protein into the cytosol

Mitochondrial membrane depolarization and release of pro-apoptotic proteins (including cytochrome c, Smac/DIABLO, AIF and HtrA2/Omi) have been implicated in both TRAIL[22] and proteasome inhibitor-induced apoptosis.[23] Since mitochondrial membrane depolarization occurs upstream of DNA fragmentation we chose an earlier time point (5 h) to investigate the percentage of cells with depolarized mitochondrial membranes. Both TRAIL (60 ng/ml) and epoxomicin (1 μM) induced some depolarization (11%±2% and 12%±3%, respectively) (*Figure 3a*). However, combination of these two drugs induced strong increase in mitochondrial membrane depolarization (44%±3%) (*Figure 3a*). These results indicated that the synergistic interaction between the two stimuli is apparent at the level of mitochondrial membrane depolarization. We studied next whether the increased membrane depolariza-





	C	C	C	C
Smac	1	1.8	1.35	4.28
Cyto-c	1	2.4	5.4	6.78
Omi	1	8.6	18.7	66.3
AIF	0	0	0	0
β-actin	1	1.48	1.67	0.62

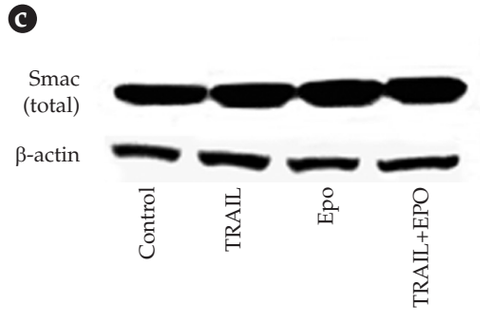


Figure 3. *Mitochondrial membrane depolarization and release of Smac/DIABLO into the cytosol is dependent on combined treatment with TRAIL and epoxomicin.* ***(a)*** *Mitochondrial membrane potential was analyzed by flow cytometry after $DiOC_6$ staining in RKO cells incubated for 5 h in the presence or absence of TRAIL (60 ng/ml) and/or epoxomicin (1 μM). Values represent the mean ± SD of three determinations. Densitometry values of the cytoplasmic fractions of each protein are represented.* ***(c)*** *In the same experiment mitochondrial (M) and cytosolic (C) fractions were separated and analyzed by Western blot for the presence of cytochrome c (cyto-c), HtrA2/Omi, Smac/DIABLO and AIF.* ***(c)*** *In the same experiment the expression of Smac/DIABLO and β-actin was determined by Western blot in whole-cell lysates of RKO cells.*

tion was accompanied by the release of pro-apoptotic mitochondrial factors into the cytosol under the same conditions. Cells were treated alone or in combination with TRAIL (60 ng/ml) and epoxomicin (1 μM) for 5 h, harvested and the mitochondrial and cytosolic fractions were isolated. Expression levels of Smac/DIABLO, HtrA2/Omi, cytochrome c (cyto-c) and AIF were analyzed by Western blot in both cytosolic and mitochondrial fractions. After 5 h the release of HtrA2/Omi and cyto-c was observed in epoxomicin- and TRAIL-treated samples. This release was further increased by TRAIL/epoxomicin co-treatment. Smac/DIABLO release occurred only in the co-treated samples (*Figure 3b*). To determine whether the increase in Smac/DIABLO in the cytosol was not a consequence of increased stability due to inhibition of proteasomal degradation, the amount of Smac/DIABLO in total cell lysates was examined in cells treated under the same conditions. No significant change in Smac/DIABLO (cellular) levels was observed in the co-treated samples in comparison to control, TRAIL or epoxomicin treated samples (*Figure 3c*). AIF was not released in these experiments. Results indicate that only co-treatment of RKO cells with TRAIL and epoxomicin results in the release of Smac/DIABLO protein into the cytosol.

TRAIL treatment leads to partial activation while TRAIL/epoxomicin co-treatment leads to full activation of caspase-3

Death receptors can directly activate caspase-3 via caspase-8, independently of mitochondrial membrane depolarization.[24] In contrast, proteasome inhibitors activate the intrinsic mitochondrial pathway by releasing cytochrome c which leads to activation of the apoptosome, caspase-9 and subsequent activation of caspase-3. Therefore, the activation of caspase-3 was next determined in lysates from RKO cells treated with TRAIL and/or epoxomicin at the same time point (5 h) with the concentrations used in the previous experiment. Caspase activity was determined (DEVDase activity) by using an artificial substrate, Ac-DEVD-AMC. Significant enzyme activity was detected only in TRAIL-epoxomicin co-treated samples after 5 h (*Figure 4a*). Caspase-3 activation was also analyzed by Western blotting using an antibody detecting pro-caspase-3 and fragments of caspase-3 generated during proteolytic activation. In control and epoxomicin-treated samples, no proteolytic caspase-3 activity was detected. TRAIL treatment alone induced partial caspase-3 activation indicated by the presence of the p20 fragment, while co-treatment with epoxomicin and TRAIL for 5 h induced full activation of caspase-3 to the p17 fragment (*Figure 4b*). These data indicate that TRAIL treatment led to only partial activation of caspase-3 in the absence of release of the mitochondrial protein Smac/DIABLO. Epoxomicin induced release of cytochrome c and HtrA2/Omi in the absence of release of Smac/DIABLO, which were insufficient to activate the intrinsic apoptotic pathway via caspase-9-mediated caspase-3 activation, since no significant caspase-3-like DEVDase activity was detected at the same conditions.

Expression levels of anti-apoptotic proteins in RKO cell

Results obtained were consistent with the inhibition of caspase-3 and caspase-9 activity by IAP proteins such as XIAP inhibiting the final auto-proteolytic activation of these caspases.[25] XIAP was found to be expressed in RKO cells by Western blot (*Figure 5*). Smac/DIABLO counteracts this inhibition and induces the complete activation of caspase-3 and caspase-9.[26] TRAIL can also induce NF-κB signaling which can lead to the upregulation of anti-apoptotic proteins including XIAP, survivin, Bcl-2, and Bcl-X_L. Proteasome inhibitors can prevent NF-κB activation by stabilizing IkB (inhibitor of κB), and this mechanism is thought to be responsible for the anti-tumor activity of proteasome inhibitors in myeloma.[27-29] Therefore, the expression levels of these proteins were determined in the pres-

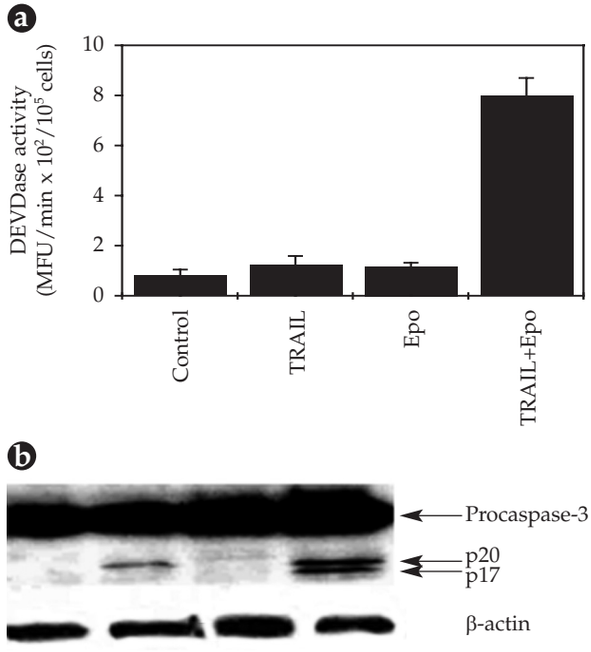


Figure 4. *Activation of caspase-3 is dependent on the combined treatment with TRAIL and epoxomicin.* ***(a)*** *Caspase-3-like activity was detected by using Ac-DEVD-AMC as the fluorogenic substrate in RKO cells incubated for 5 h in the presence or absence of TRAIL (60 ng/ml) and/or epoxomicin (1 μM) and presented as a relative value compared to control activity. Values indicate the mean±SD of three determinations. (b) Proteolytic activation of caspase-3 and expression of β-actin were analyzed in the same experiment by Western blot.*

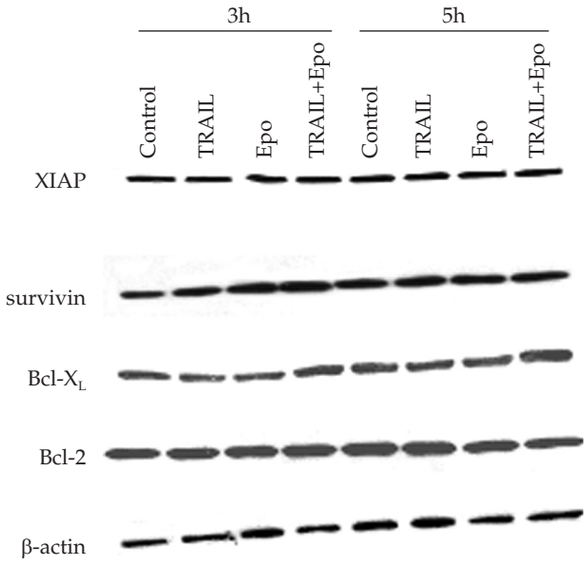


Figure 5. *Expression of anti-apoptotic proteins. Expression of XIAP, survivin, Bcl-X_L, Bcl-2, and β-actin were analyzed by Western blot in RKO cells incubated for 5 h in the presence or absence of TRAIL (60 ng/ml) and/or epoxomicin (1 μM).*

ence and absence of TRAIL and proteasome inhibitors after 3-h and 5-h incubation. No change in expression levels of these proteins was detected under any of these conditions (*Figure 5*). Results indicate that although NF-κB regulated anti-apoptotic genes such as XIAP are expressed in RKO cells, inhibition of NF-κB signaling by proteasome inhibition does not contribute to the enhanced induction of apoptosis in these cells treated with TRAIL and epoxomicin.

Discussion

In this paper we have provided data for a new mechanism to describe how proteasome inhibitors and TRAIL induce synergistic cell death in colon carcinoma cells. We also presented new data about the regulation of the release of pro-apoptotic proteins from the mitochondria.

Previously, we determined that most colon carcinoma cell lines are resistant to TRAIL-induced apoptosis despite the expression of TRAIL receptor DR5.[29] The ability of proteasome inhibitors to increase TRAIL-induced apoptosis has been reported in other cell types.[15,30-34] We have found that proteasome inhibitors at sub-cytotoxic concentrations effectively sensitize resistant colon carcinoma cell lines to TRAIL, and also other cell lines of various origin (rhabdomyosarcoma, ovarian carcinoma; unpublished data). RKO cells utilized in the current study were completely resistant to TRAIL or the proteasome inhibitors (MG132, PS-341 and epoxomicin) administered alone, however, the combination treatment of TRAIL with an individual proteasome inhibitor significantly induced apoptosis as determined by an increase in the sub-G1 population after 6-h incubation reaching a plateau at 10 h. The general caspase inhibitor z-VAD-fmk completely abolished the increase in sub-G1 cells, consistent with a mechanism dependent on the caspase-3-dependent activation of CAD DNAse.[35,36] z-IETD-fmk, a relatively selective inhibitor of caspase-8, significantly but not completely inhibited DNA fragmentation. Caspase-8 is believed to play a central role in death receptor-induced apoptosis,[37] however, evidence of a caspase-8-independent component in TRAIL-induced activation of Bax and cell death has been reported. [6]

Previously, we and others have found that overexpression of Bcl-2 or Bcl-X_L inhibits TRAIL-induced apoptosis (as well as activation of Bax) implicating a role of the mitochondrial pathway in TRAIL-induced apoptosis.[6,38,39] Proteasome inhibitors can induce apoptosis dependent on functional Bak and Bik or Bim.[18-20] Furthermore, Bortezomib/PS-341-induced cell death is impaired in the absence of Apaf-1.[20] We found that the combination of TRAIL and epoxomicin induced significant mitochondrial membrane depolarization while these treatments individually induced only a very small increase in the percentage of cells with decreased mitochondrial membrane potential.

Johnson et al. have found that bortezomib/PS-341 increased the expression of DR5.[17] According to their model, increased expression of DR5 led to increased activation of caspase-8 and increased cleavage and activation of Bid in response to TRAIL treatment.[40] Truncated Bid is known to activate Bax or Bak, which leads to the release of cytochrome c, Smac/DIABLO, HtrA2/Omi and AIF. Apoptosis induced by the combined treatment of TRAIL and bortezomib/PS-341 was dependent on the expression of Bak in MEF cells.[17]

We have determined that the expression of DR5 in RKO cells increased after epoxomicin treatment as measured by flow cytometry (data not shown). However, a more complex interaction between proteasome inhibitors and TRAIL is demonstrated in our experiments. In RKO cells TRAIL alone induced partial activation of caspase-3 in the absence of mitochondrial membrane depolarization. However, caspase-3 was not cleaved into its most active form, as indicated by the absence of the p17 cleavage product. This observation is also supported by the low DEVDase (caspase-3-like) activity and the absence of DNA fragmentation. The proteasome inhibitor epoxomicin administered alone induced the release of cytochrome c and HtrA2/Omi without subsequent activation of caspase-3 or induction of DNA fragmentation. TRAIL alone did not induce the release of any of these mitochondrial proteins. However, combination treatment of TRAIL and epoxomicin induced the release of Smac/DIABLO from the mitochondria, while release of

cytochrome c or HtrA2/Omi was unchanged compared to epoxomicin treatment alone. Total expression of Smac/DIABLO in the whole cell lysate remained unchanged and AIF was not released under any treatment conditions, supporting the selective regulation of the release of individual pro-apoptotic proteins from the mitochondria. The release of Smac/DIABLO was paralleled by the full activation of caspase-3 from the p20 to the p17 fragment, increased DEVDase (caspase-3-like) activity and subsequent DNA fragmentation. These results are consistent with a model where IAP proteins, e.g. XIAP or c-IAP1/2, which are constitutively expressed in this cell line, bind to procaspase-3 and procaspase-9 and inhibit their full proteolytic activation.[6] In this case, both the extrinsic pathways and the proteasome-induced mitochondrial intrinsic pathway are blocked. Smac/DIABLO can dissociate IAP proteins from caspases thereby opening possibility for full caspase activation.[26] It is important to note that the model explains why cells with caspase-9 deficiency[17] or Apaf-1 null cells[20] are sensitive to the combined treatment of TRAIL and proteasome inhibitors. Furthermore, proteasome inhibitors not only sensitize TRAIL-resistant cells to TRAIL-induced cell death, but the direct activation of caspase-3 combined with release of Smac/DIABLO via death receptors sensitize cells to proteasome inhibitors, which are otherwise resistant due to the absence of caspase-9, Apaf-1 or inhibition by Akt kinase.

The molecular mechanism that leads to the enhanced, almost selective release of Smac/DIABLO from the mitochondria is not fully understood. Recently, two independent groups reported that PS-341 and other proteasome inhibitors (MG132 and ALLN) can upregulate the level of the BH3-only proteins Bik and Bim.[18-20] Accumulation of these pro-apoptotic proteins was due to decreased proteasomal degradation in the presence of proteasome inhibitors. Downregulation of Bik with siRNA identified this mechanism as the primary cytotoxic action of PS-341 in cancer cell lines including colorectal cancer cells.[18] Bik tanslocates to the mitochondria and induces permeability transition, leading to the release of cytochrome c.[41] This in turn activates the formation of the apoptosome and activation of caspase-9 and downstream effector caspases such as caspase-3.[42] These results established the model where PS-341 mainly acts via the mitochondrial pathway. The same model was applied when PS-341 mediated sensitization to TRAIL was analyzed. It was suggested that increased level of Bik can "titrate out" anti-apoptotic Bcl-2 family members leading to increased mitochondrial membrane depolarization and release of the pro-apoptotic proteins cytochrome c and Smac/DIABLO in response to TRAIL treatment.[18,20] This model, however, is not fully consistent with our results. We cannot rule out the possibility that a special interaction between Bid and Bik, or between the partially activated caspase-3 and Bik leads to mitochondrial membrane depolarization and release of Smac/DIABLO via a Bax- or Bak-dependent mechanism (*Figure 6*).

RKO cells express Bcl-2, which can also explain resistance to both TRAIL and proteasome inhibitors. Similarly to previous reports,[6] the overexpression of Bcl-2 or Bcl-X_L in colon carcinoma or TRAIL-sensitive rhabdomyosarcoma (RMS) cell lines inhibited TRAIL- or proteasome inhibitor-induced cell death, however, the combined treatment was still very effective (not published). It is difficult to explain this phenomena with our current model since it is unlikely that Bik alone can "titrate out" artificially high transgene expressions. Furthermore, although Bik -/-, Bim -/- double knock-out MEF cells were resistant to bortezomib/PS-341 or TRAIL alone, they were equally sensitive to the combination treatment in comparison to wild-type cells.[20] Recently, the degradation of overexpressed

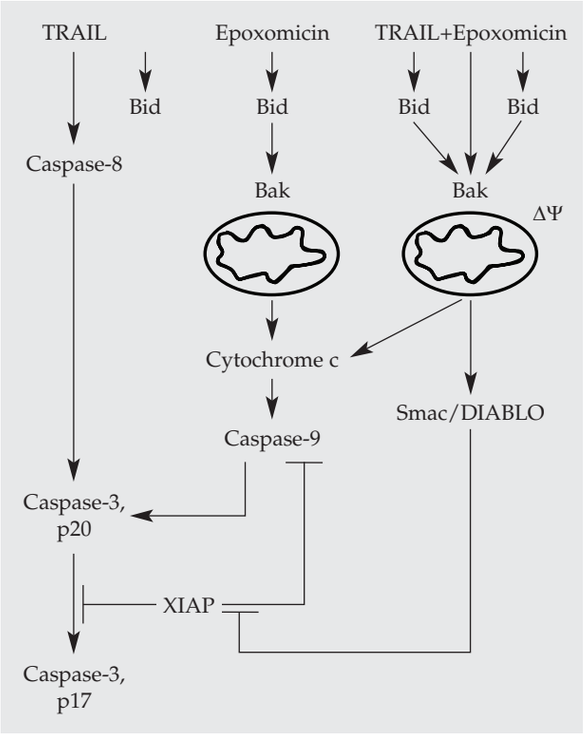


Figure 6. *Model for the molecular interaction between TRAIL and proteasome inhibitors in colon carcinoma cells. TRAIL directly activate caspase-3 via caspase-8, however, IAP proteins prevent the full proteolytic activation of caspase-3. Epoxomicin upregulates Bik and induces the release of cytochrome c in the absence of mitochondrial membrane depolarization and the release of Smac/DIABLO. In the absence of Smac/DIABLO caspase-9 activation is inhibited by XIAP. Combined treatment cooperates by a Bik- and Bak-dependent mechanism to induce mitochondrial membrane depolarization and release of Smac/DIABLO which leads to the full activation of caspase-3.*

Bcl-2 and Mcl-1 was reported in Jurkat cells in response to the combination treatment with bortezomib/PS-341 and TRAIL.[43] However, no degradation of Bcl-2 was observed in colon carcinoma cells, and Mcl-1 is known to be a specific survival factor in lymphoid cells.[44] Previously, we suggested the presence of a caspase-8/Bid-independent, but FADD-dependent signaling mechanism in TRAIL-induced apoptosis in human RMS cells.[6] It is possible that such a mechanism could lead to increased sensitivity to mitochondrial membrane depolarization without activating the Bax-dependent caspase-8-Bid pathway.

Zhu et al did not find changes in IkBá levels after PS431 treatment, which downplays the significance of inhibition of NF-κB signaling in proteasome activity in colon carcinoma cells. However, TRAIL can activate the NF-κB pathway in some cell types and this induced signaling can be inhibited by proteasome inhibitors.[15] In RKO cells the level of anti-apoptotic proteins XIAP, survivin, Bcl-X_L or Bcl-2 regulated by NF-κB did not change after TRAIL or proteasome inhibitor treatment, which makes unlikely a significant role for NF-κB inhibition as a mechanism for the synergistic interaction between the proteasome inhibitors and TRAIL in these colon carcinoma cell lines.

The current model suggests that the death receptor-induced signaling mechanisms involving activation of Bid and partial activation of caspase-3 synergize with PS-341-induced Bak and possibly Bik pathways in the enhanced release of Smac/DIABLO from the mitochondria. The model also suggests that proteasome inhibitors can trigger cell death by the "type II/b" signaling pathway,[45] where mitochondrial release of Smac/DIABLO opens the way for the direct activation of pro-caspase-3 (type-I) by death receptors leading to cell death as an alternative mechanism of the activation by a functional apoptosome.

The detailed, tumor type-specific understanding of molecular mechanisms responsible for the synergism between TRAIL or agonistic antibodies of TRAIL receptors and proteasome inhibitors may help better design clinical trials and develop diagnostic methods for individualized therapy.

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