Chapter 4

TRAIL therapy in non-small cell lung cancer cells: sensitization to death receptor mediated apoptosis by proteasome inhibitor bortezomib

Jens Voortman, Tatiana P Resende, Mohamed AI Abou El Hassan, Giuseppe Giaccone and Frank AE Kruyt

Molecular Cancer Therapeutics 2007, 6:2103-12

Abstract

Activation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor pathway is a promising therapeutic strategy to selectively eradicate cancer cells, including non-small cell lung cancer (NSCLC) cells. Recombinant human (rh) TRAIL/ Apo-2L, a TRAIL-encoding adenovirus (AdTRAIL) and monoclonal antibodies directed against the TRAIL-receptors R1 and R2 were used to study cytotoxicity of TRAIL-therapy in NSCLC cells. NSCLC cells showed differential sensitivity to TRAIL-therapy, regardless of the agent used. Combination treatment of bortezomib and rhTRAIL led to synergistic apoptosis induction in NSCLC cell lines. Enhancement of rhTRAIL-induced apoptosis by bortezomib was caspase-dependent, implicating extrinsic as well as intrinsic apoptosis activation, as shown by increased processing of caspase-8 as well as caspase-9, and could be abrogated completely by overexpression of caspase-8 inhibitor CrmA, and partially by overexpression of Bcl-2. Enhanced surface expression of TRAIL-R2, but also TRAIL-R1, was associated with bortezomib treatment, which is likely to contribute to the increased processing of caspase-8 in the combination treatment. Furthermore, TRAIL-induced activation of pro-survival transcription factor NF-κB was prevented by co-treatment with bortezomib, which may contribute to the observed synergistic apoptosis induction. Our preclinical data indicate that combination therapy of TRAIL and bortezomib may be an effective strategy for NSCLC.

Introduction

The basic apoptotic machinery can be activated through two distinct pathways. The mitochondrial or so-called "intrinsic" apoptotic pathway involves mitochondrial outer membrane permeabilization (MOMP), regulated by the Bcl-2 family of proteins, and subsequent release of pro-apoptotic factors, e.g. cytochrome-c and Smac/ DIABLO, formation of the apoptosome and activation of initiator caspase-9 [Green and Reed, 1998; Cain et al., 1999]. The death receptor (DR) pathway, or "extrinsic" apoptotic pathway, is triggered by membrane-bound death receptors, which, after binding to their ligands, recruit several intracellular proteins to their cytosolic domain, forming the "death-inducing signalling complex" (DISC), responsible for activating initiator caspase-8 [Wallach et al., 1999].

The tumor necrosis factor (TNF) family consists of proteins involved in proliferation, differentiation and apoptosis [Krammer, 1998]. Examples of members of this family are TNF-α, CD95L and TNF- related apoptosis-inducing ligand (TRAIL, also known as Apo-2L) [Suliman et al., 2001]. Contrary to other TNF family members, TRAIL is expressed in a broad range of tissues and exerts great antitumor activity, selectively inducing apoptosis in tumor cells, sparing normal cells [MacFarlane, 2003; Wang and El-Deiry, 2003]. Systemic administration of TRAIL reduced mammary adenocarcinoma growth in mice without any of the toxic effects, notably no hepatotoxicity, observed when using FasL or TNF-α [Suliman et al., 2001]. Therefore, TRAIL might constitute a promising anti-cancer agent and currently clinical studies are being conducted with rhTRAIL as well as agonistic monoclonal antibodies directed against the TRAIL-R1 and -R2 receptors [Tolcher et al., 2004; Ling et al., 2006; Patnaik et al., 2006].

TRAIL can bind to five receptors, of which four are located at the cell surface: TRAIL-R1 (DR4), TRAIL-R2 (DR5/ KILLER), TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) [Wang and El-Deiry, 2003] . Besides the TNF homology domain (THD), they possess the TRAIL-binding site (TBS). When a specific TRAIL-receptor trimerizes, it can bind via the TBS to the TRAIL ligand [Bodmer et al., 2002]. A soluble receptor called osteoprotegerin (OPG) can also bind TRAIL but with lower affinity [Wang and El-Deiry, 2003]. Only two of these receptors, R1 and R2, contain a functional cytoplasmatic death domain (DD) motif and are capable of delivering TRAIL's apoptotic signal by association of the DD with the

FAS-associated death domain (FADD) protein, containing the death effector domain (DED), which is involved in the activation of caspase-8 [Suliman et al., 2001]. TRAIL can bind with similar affinity to R1 and R2 [Schneider et al., 1997]. The other two receptors, DcR1 and DcR2, are called "decoy receptors", lacking the ability to initiate the apoptotic cascade. DcR2 contains a truncated DD while the DD of DcR1 lacks a cytoplasmatic domain [Wang and El-Deiry, 2003].

A correlation between TRAIL sensitivity and DR4, DR5 and/ or decoy receptor expression has been claimed in some cell lines [Georgakis et al., 2005; Khanbolooki et al., 2006]. For example, normal cells were reported to express high levels of DcR receptors and low levels of the R1 receptor [Kim et al, 2000]. It was shown that removal of surface DcR1 by phosphatidylinositol-specific phospholipase-C could overcome resistance to TRAIL-induced apoptosis [Zhang et al., 2000]. However, others claimed expression of decoy receptors does not explain TRAIL resistance [Griffitih et al., 1999]. Furthermore, DcR2 might rather be a regulatory receptor instead of a decoy receptor. Whereas most TNF receptors normally form only homotrimeric complexes, preligand assembly domains in TRAIL-R2 and TRAIL-R4 permit mixed complex formation, thus regulating apoptosis induction, without the requirement of ligand binding by DcR2 [Clancy et al., 2005].

Different studies relate resistance to TRAIL-induced cell death to downstream factors. It has been shown that down-regulation of cellular FLICE-like inhibitory protein (c-FLIP) can sensitize cells to TRAIL-induced apoptosis [Siegmund et al., 2002; Okano et al., 2003]. Overexpression of Bcl-2 was related with inhibition of TRAIL-induced apoptosis [Fulda et al, 2002; Abou El Hassan et al, 2004]. Also, it has been shown that resistant cells can become sensitive when treated with chemotherapeutic agents [Griffitih et al., 1999]. Recently, reports show a sensitization to TRAIL-induced apoptosis in a variety of tumor cell types by combining TRAIL with proteasome inhibitors, e.g. bortezomib (Velcade™) [Johnson et al., 2003; Zheng et al., 2004; Georgakis et al., 2005; Khanbolooki et al., 2006]. The mechanism of sensitization seems to differ among cell types but typically involves increased caspase-8 activation with or without a decrease in the level of c-FLIP. Furthermore, a bortezomib-induced increase in the levels of BH3-only pro-apoptotic Bcl-2 proteins such as Bik and Bim, as well as inhibition of (TRAIL-induced-)NF-κB activation may

play a role [Keane et al., 2000; Johnson et al 2003; Sayers et al., 2005; Nikrad et al., 2005; Khanbolooki et al., 2006].

In this study, we evaluated susceptibility to TRAIL therapy in NSCLC cells and analyzed the expression of the different TRAIL receptors. We observed synergistic apoptosis induction when rhTRAIL was combined with proteasome inhibitor bortezomib. Molecular events underlying this synergistic apoptosis induction were investigated.

Materials and methods

Cell Lines and Drugs

NSCLC cell lines H460, A549, SW1573, H292, H1299 and H322 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). We have previously generated stably transfected derivatives of H460 cells, overexpressing Bcl-2 (H460-Bcl-2) and caspase-8 inhibitor CrmA (H460-CrmA) [Ferreira et al, 2000]. SW1573 cells were cultured in DMEM tissue culture medium (Cambrex Bioscience, Verviers, Belgium). H460, A549, H292, H1299 and H322 cells were cultured in RPMI 1640 medium (Cambrex Bioscience). Culture mediums were supplemented with (v/v) 10% FCS, 100 units/ ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Breda, The Netherlands). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The replication incompetent adenovirus-encoding TRAIL (AdTRAIL) was constructed as described before [Abou El Hassan et al, 2004]. Briefly, the TRAIL open reading frame (ORF) was provided by Dr. H. Yagita (Juntendo University School of Medicine, Japan) and cloned under the control of a cytomegalovirus (CMV) promoter in a pShuttle plasmid. In vitro recombination of pShuttle TRAIL with the pAdeasy plasmid allowed the production of AdTRAIL. The adenovirus vectors were propagated in 293 cells and purified by CsCl density gradient. Monoclonal antibodies (mAbs) against the TRAIL receptors (TRAIL-R1: huTR1-M271; TRAIL-R2: huTRAILR2-M413; TRAIL-R3: huTRAILR3-M430; TRAIL-R4: huTRAILR4-M440) were kindly provided by Amgen Inc. (Seattle, WA). Monoclonal antibodies against TRAIL receptors R3 and R4 (clones HS301 and HS402) (Alexis, Lausen, Switzerland). Recombinant human TRAIL/ Apo-2L (rhTRAIL) (PeproTech EC Ltd, London, UK) was dissolved in phosphate buffered saline and Helenalin (Biomol, Plymouth Meeting, PA) in DMSO. Bortezomib (Velcade™) (formerly PS-341) was kindly provided as a pure substance by Millennium Pharmaceuticals Inc. (Cambridge, MA) and dissolved in DMSO. When applicable cells were pretreated for 2h with the synthetic pancaspase inhibitor z-VAD-fmk (Enzyme System Products, Livermore, CA).

Growth-Inhibition Assay

The cytotoxic effect of TRAIL-receptor-directed monoclonal antibodies (mAbs), AdTRAIL, rhTRAIL, and of bortezomib in combination with rhTRAIL or AdTRAIL, was determined by

incubating cells at a density of $1x10^3$ per well of 96-well flat-bottomed plates (Costar, Corning, NY) 24h prior to treatment. In AdTRAIL studies, cells were incubated with different multiplicity of infection (MOI) i.e., virus to cell ratios of AdTRAIL (range: 0-200). The infection procedure was as described before [Abou El Hassan et al, 2004]. Cells were incubated with 50 µl/well of different MOIs at 37 ºC. Two hours after infection, the same volume of virus-free medium was added to each well. In the TRAIL-R1/2 mAb and rhTRAIL studies, cells were incubated with 100µl/well of antibodies (range: 0-30 µg/ml) or rhTRAIL (range: 9.4-300 ng/ml). For the combination studies with bortezomib, a fixed dose of TRAIL (50 ng/ml) was combined with a concentration range of bortezomib (3-500 nM). Cell viability was determined 24h (combination rhTRAIL/ bortezomib), 48h (agonistic TRAIL mAbs; rhTRAIL; combination AdTRAIL/ bortezomib) or 72h after (AdTRAIL), by MTT assay, as described previously [Abou El Hassan et al, 2003]. Briefly, cells were washed twice with PBS. Next, 50 µl of 1mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma co., St. Louis, MO) solution was added to each well, followed by a 2h incubation at 37ºC. The formazan crystals were dissolved using 150µl of dimethyl sulphoxide (DMSO) and absorbance was measured at 595nm on a Spectra Fluor multiwell plate reader (Tecan, Salzburg, Austria). Results are presented as percentage of survival taking the control (untreated cells) as 100% survival. The concentration resulting in 50% of cell-growth inhibition (IC_{50}) was calculated using SigmaPlot version 8.0 (SPSS Inc., Chicago, IL).

Measurement of mRNA Transcript Levels of TRAIL receptors

Total RNA was isolated from the human cell lines with TRIzol reagent (Invitrogen) according to the manufacturer´s instructions. RNA samples (5µg) were treated using a DNA-free RNA kit (Zymo Research, Orange, CA). cDNA synthesis was performed using a RNA PCR kit containing an oligo$(dT)_{20}$ primer (Invitrogen). Reverse transcription was performed using a thermal program of 50ºC for 60 min and 85ºC for 5 min. PCR reactions were performed using the primers published by Griffith *et al.*, 1998 [Griffith et al, 1998]:(TRAIL-R1 (forward: 5´-CTGAGCAACGCAGACTCGCTGTCCAC-3´; reverse: 5´-TCCAAGGCACGGCAGAGCCTGTGCCAT-3´),TRAIL-R2 (forward: 5´-GCCTCATGG ACAATGAGATAAAGGTGGCT-3´;reverse:5´-CCAAATCTC AAAGTACGCACAAAC GG-3´),TRAIL-R3 (forward: 5´-GAAGAATTTGGTGCCAATGCCACTG-3´ ; reverse: 5´-CTCTTGGA CTTGG CTGGGAGATGTG -3´) and TRAIL-R4 (forward: 5´-CTTTTCCGGCGGCGTTCATGTCCTTC-3´; reverse: 5´-GTTTCTTCCAGGCTGC TTCCCTTTGTAG-3´) giving products of 506, 502, 612 and 453 bp, respectively. A hot-start program was made for all primers, 95º C for 5 min and 80ºC for 10 min. PCR cycle conditions were 94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min, for 30 cycles. Samples were resolved on a 1% agarose gel and visualized with ethidium bromide.

Measurement of Surface Expression of TRAIL-receptors

Five million cells, untreated or treated with bortezomib (100nM for 16h) (H460 cells only) were added to a FACS tube and stained with receptor-specific mAbs for 1 hour at 4º C. To control for non-specific binding, an IgG1 antibody (DAKO) for R1 and R4 and an IgG2a antibody (DAKO) for R2 and R3 were used as isotype-matched nonbinding antibodies. One microgram of each of the specific antibodies or the respective isotype antibody was used for each sample. After incubation with the first antibody, cells were washed twice with cold PBS/ 0.5% BSA and incubated with FITC-labelled secondary antibody (DAKO) at a dilution of 1:50 for 30 min at 4º C in the dark. Cells were washed twice with cold PBS/ 0.5%BSA before analysis by FACScalibur using CELLQuest software (Becton Dickinson, Mountain View, CA).

Cell Death Measurement

Propidium iodide (PI) staining and flow cytometry analysis were performed as described previously [Ferreira et al., 2000]. The fraction of cells with hypodiploid (sub-G_1) DNA content, was taken as the apoptotic cell population. The percentage of apoptosis indicated was corrected for background sub-G_1 levels found in the corresponding untreated controls.

Western blot analysis

Western blot analysis was performed as described before [Ferreira et al., 2000]. Rabbit polyclonal primary antibodies used were: anti-caspase-9, anti-caspase-3, anti-PARP, anti-c-Flip, anti-Bid (Cell Signaling Technology, Beverly, MA), anti-caspase-8 (Alexis), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse monoclonal primary antibody used were anti-NOXA (Calbiochem, San Diego, CA), anti-p53 (DAKO, Santa Barbara, CA), anti-XIAP (MBL, Nagoya, Japan). As secondary reagents, horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse antibodies (Amersham, Braunschweig, Germany) were used.

NF-κB activity luciferase assay

The NF-κB reporter construct (pNF-κB) (Clontech, Mountain View, CA) containing the firefly luciferase (luc) gene from *Photinus pyralis* and four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter (P_{TAL}) region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter was used. After endogenous NF-κB proteins bind to the kappa (κ) enhancer element, transcription is induced and the reporter gene is activated. Fifteen thousand H460 cells were plated in each well of 96-well flat-bottomed plates and incubated until 60–80% confluency. Cells were co-transfected with pNF-κB (1.0 μg/ml) and, for normalization, pRL-CMV (0.1 μg/ml) (Promega, Madison, WI) using the SuperFect transfection reagent (QIAGEN, Venlo, The Netherlands). Following transfection, cells were cultured with either bortezomib (100 nM), rhTRAIL (50ng/ml), alone or in combinations for 13h, at which time luciferase activity was measured.

Luciferase activities in cell lysates were measured using the Dual Luciferase assay system (Promega) and a Luminat LB9507 luminometer (EG&G Gerthold, Bad Wildbad, Germany). Results were expressed as relative light units (RLU) normalized for Renilla luciferase activity. The results are presented as the fold induction, which is the relative luciferase activity of the treated cells over that of control cells. All transfection experiments were carried out in triplicate wells and repeated separately at least three times.

Results

The cytotoxic effects of TRAIL therapy in NSCLC cells

A commonly used strategy to activate the TRAIL-receptor pathway is exposing cells to soluble rhTRAIL/ Apo-2L, a 168 amino acid polypeptide (19.6 kDa), consisting of the TNF homologous portion of the extracellular domain of the full length TRAIL protein. Many studies have evaluated cytotoxicity of rhTRAIL in tumor cell lines, including NSCLC cells, which are generally quite resistant to rhTRAIL-induced cytotoxicity [Yeow et al., 2006; Jin et al., 2007].

Here we studied alternative, potentially more effective, strategies to activate TRAIL-receptor mediated apoptosis. Figure 1A shows the growth curves of NSCLC cells treated with TRAIL-R1- and -R2 directed monoclonal antibodies. Antibodies have higher binding affinity and less reactivity towards decoy receptors, which can lead to an altered cytotoxicity profile compared to rhTRAIL [Finnberg et al, 2006]. The IC_{50} values for each antibody were determined (Figure 1C) showing no differential sensitivity to either antibody. We then asked the question whether the relative resistance of some NSCLC cell lines, e.g. A549 and SW1573, to the antibodies could be referred to the sole activation of one death receptor instead of dual receptor activation and therefore cells were infected with a *full-length* TRAIL-encoding adenovirus. Full-length TRAIL has been reported to induce a media-transferable bystander effect and was shown to induce apoptosis in rhTRAIL-resistant cell lines, including NSCLC cell lines [Voelkel-Johnson et al., 2002; Seol et al., 2003]. Figure 1B shows the growth curves for NSCLC cells infected with AdTRAIL. IC_{50} values corresponding to the multiplicity of infection (MOI) of AdTRAIL necessary to kill 50% of the cells were calculated (Figure 1C). The comparative sensitivity of the tested cell lines to the full-length TRAIL was comparable to the antibodies and the viability of the A549 and SW1573 cells was hardly affected. Next, we examined whether H460 and SW1573 cells

displayed a similar differential sensitivity when exposed to soluble rhTRAIL. Indeed, H460 cells were more sensitive than SW1573 cells to both the growth inhibitory and apoptosis-inducing effects of rhTRAIL (Figure 1D).

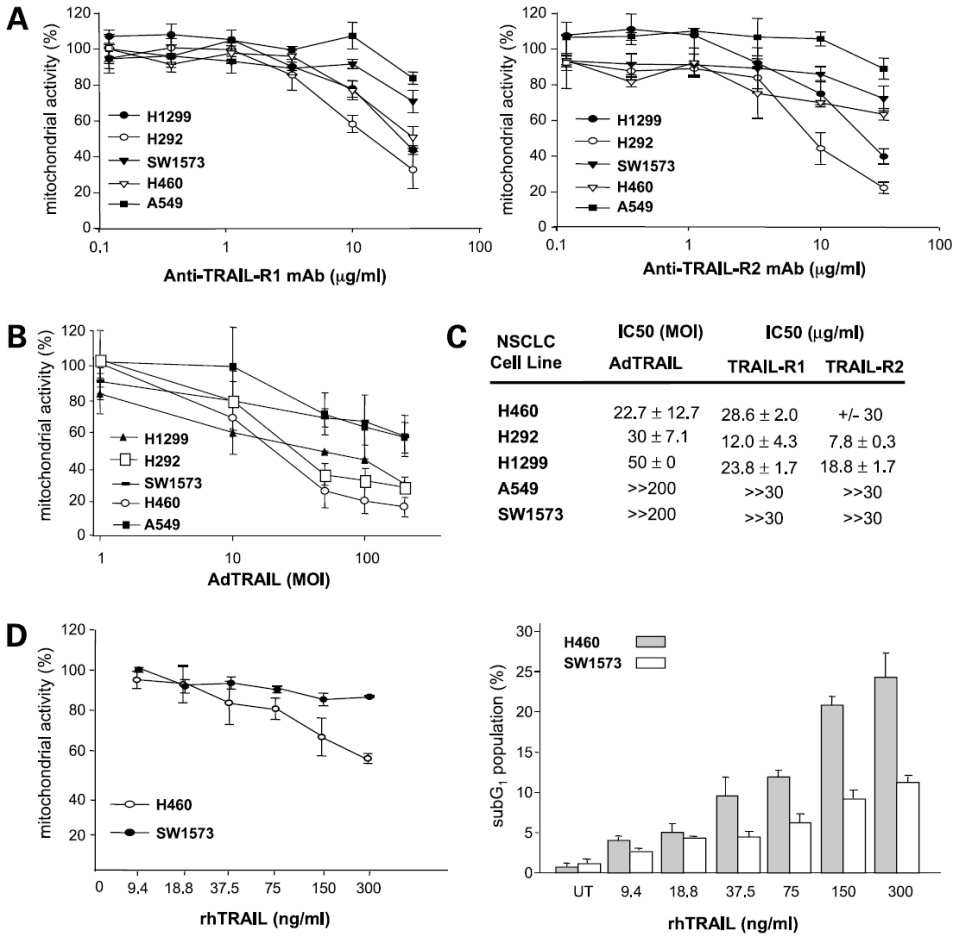


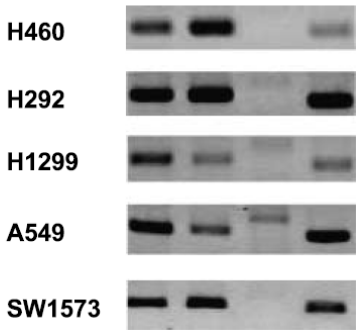
Figure 1. Cytotoxic effects of TRAIL therapy in NSCLC cells. A, growth curves (MTT assay) of NSCLC cells treated with TRAIL-R1 and -R2 directed monoclonal antibodies. **B,** growth curves (MTT assay) of NSCLC cells infected with a full-length TRAIL-encoding virus (AdTRAIL). **C,** calculated IC_{50} values for the different drugs in the different cell types. **D,** growth curves (MTT assay) and estimated subG1 population by PI staining-based FACS analysis of H460 and SW1573 cells treated with rhTRAIL/Apo-2L. Means of triplicate determinations ± SD.

TRAIL receptors: mRNA levels and surface expression.

A possible mechanism of the differential sensitivity of the tested cells to TRAIL-induced apoptosis could be due to the variability of the cell surface

levels of the death and decoy receptors resulting in increased apoptotic signalling in the sensitive cells. We next investigated whether levels of receptor expression could explain the differences in TRAIL sensitivity in NSCLC cell lines. Figure 2A shows the messenger RNA levels of the different receptors as measured by RT-PCR. In general death receptors are expressed at comparable levels in all cell lines. The expression of R3 was marginal in most cells and absent in H460 and SW1573 cells. The expression of R4 receptor was low in H460 and H1299 compared to the rest of cells. Thus TRAIL sensitive cells could not be differentiated from the resistant cells based on the mRNA levels of the death and decoy receptors. FACS analysis was used for quantitative determination of cell surface TRAIL-receptor expression on the cell surface using the Amgen monoclonal antibodies against human TRAIL receptors (Figure 2B). The cut-off value for the ratio between specific antibodies and the isotype antibodies was set at 1.5. All the NSCLC cell lines tested are positive for both TRAIL-R1 and -R2. Resistant cell line SW1573 showed comparable death receptor levels as H460, H292, H1299 cells, with higher expression than in A549 cells. We could not demonstrate DcR surface expression in any of the cell lines tested using the antibodies from Amgen nor using TRAIL-receptor-directed mAbs from a different supplier (Alexis Inc.). Thus TRAIL sensitive cells could not be differentiated from resistant cells based on the surface expression of the death and decoy receptors.

A



B

Cell Line	R1	R2
H460	6.8 ± 0.2	2.6 ± 0.2
H292	2.7 ± 0.1	2.9 ± 0.2
H1299	3.7 ± 0.2	2.8 ± 0.4
A549	1.7 ± 0.1	1.9 ± 0.1
SW1573	3.5 ± 0.1	4.0 ± 0.3

Figure 2. TRAIL receptor expression. ***A,*** mRNA transcripts levels, as determined by RT-PCR, of the four different TRAIL receptors in NSCLC cells. ***B,*** TRAIL receptor surface expression as determined by FACS analysis. Values correspond to a ratio of the signal of the specific TRAIL-receptor antibody and the negative isotype matched control antibody. Surface expression is considered significant when the ratio is at least 1.5. Means of triplicate determinations ± SD.

Combination therapy with the proteasome inhibitor Bortezomib

To evaluate whether co-incubation with proteasome inhibitor bortezomib can sensitize NSCLC to rhTRAIL-induced apoptosis, sensitive H460 and resistant SW1573 cells were exposed for 24h to various concentrations of bortezomib (3-500nM) in presence or absence of a fixed concentration of rhTRAIL (50ng/ ml). Furthermore, as a control, samples were treated with rhTRAIL (50ng/ ml) only. Figure 3A shows the growth curves for the two cell lines treated with bortezomib or bortezomib and rhTRAIL. We then determined the "predicted" growth inhibition curve of the combination of the two drugs by fractional effect analysis [Peters et al., 2000]. The predicted growth inhibition curve indicates the curve obtained in case of additive growth inhibition by combining bortezomib and rhTRAIL. At each concentration of bortezomib the relative growth inhibitory effect of treatment with only TRAIL (50ng/ ml) was multiplied with the relative effect of treatment with only bortezomib [Peters et al, 2000]. An actual growth inhibition curve inferior to the predicted curve indicates synergistic growth inhibition. We observed synergistic growth inhibition at 50 nM or higher concentrations of bortezomib combined with TRAIL (50ng/ ml). This may be related to the level of proteasome activity inhibited. Previously we showed the proteasome activity to be reduced to 25% of its basal level when H460 cells were treated with 100nM bortezomib. However, the proteasome activity was not significantly inhibited when cells were treated with only 10nM [Voortman et al, 2007]. Also, in a proof of principle experiment, the growth inhibitory effect of combining AdTRAIL (MOI 10) and a concentration range of bortezomib in H460 cells was examined, showing a similar synergistic activity at higher bortezomib concentrations (Figure 3B). Figures 3C-D show the apoptosis-inducing activity of bortezomib and rhTRAIL alone, or upon simultaneous exposure in NSCLC cells. Figure 3C shows in H460 cells that the simultaneous treatment of bortezomib and rhTRAIL results in a synergistic increase of the apoptotic subG_1 population, as determined by PI-staining based FACS analysis, starting at 16 hours of exposure. Similar profiles were detected in other NSCLC cell lines as graphically depicted in figure 3D for A549, SW1573 and H322 upon 24 hour treatment, including the PI-staining profiles of A549 cells. The majority of untreated cells are typically in the G_1 phase of the cell cycle. In contrast to treatment with rhTRAIL, exposure to bortezomib results in a G_2/M arrest of treated cells.

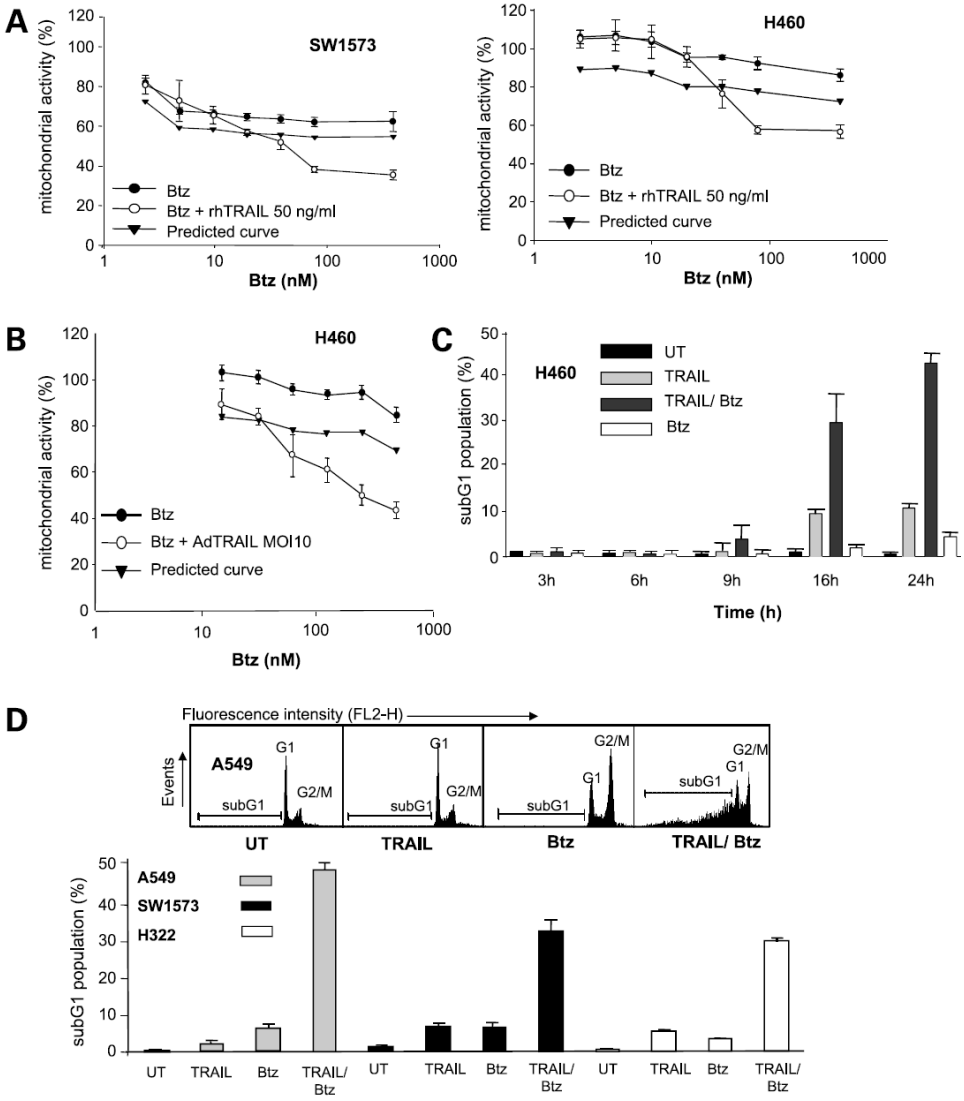


Figure 3. Combination treatment of rhTRAIL or AdTRAIL and bortezomib. A, growth- curves (MTT assay) of SW1573 and H460 cells treated with a concentration range of bortezomib, with or without the addition of rhTRAIL (50ng/ml). Included in the figures are the predicted growth curves of the combination of both drugs, based on fractional effect analysis. **B,** growth curve of H460 cells treated with a concentration range of bortezomib, with or without addition of AdTRAIL (MOI 10). The predicted growth-inhibition curve is also included. **C,** the $subG_1$ fraction determined by PI-staining-based FACS analysis is shown for H460 cells treated with rhTRAIL (50ng/ml), bortezomib (100nM), or the combination of both drugs for various periods of time. ***D,*** $subG_1$ fraction, estimated by PI-staining-based FACS analysis, of A549 (including PI staining profile), SW1573 and H322 NSCLC cells treated with rhTRAIL (50 ng/ml), bortezomib (100nM) or the combination of both drugs, for 24h. Means of triplicate determinations, SD.

Synergistic bortezomib-rhTRAIL induced apoptosis is abrogated by caspase-8 inhibition

To further study the molecular events underlying the observed enhancement of apoptosis by the combination treatment of rhTRAIL and bortezomib, we made use of two previously established H460-derived cell lines: H460-Bcl-2, stably overexpressing exogenous anti-apoptotic protein Bcl-2, an inhibitor of the mitochondrial apoptotic pathway, and H460-CrmA, stably overexpressing exogenous caspase-8 inhibitor cytokine response modifier A (CrmA), an inhibitor of the extrinsic apoptotic pathway. As shown in Figure 4A, stable overexpression of Bcl-2 abrogated rhTRAIL-induced apoptosis induction and partially abrogated apoptosis induced by bortezomib and the combination of both drugs. CrmA overexpression resulted in complete inhibition of apoptosis induction by rhTRAIL and the combination of both drugs. When H460 cells were pre-treated with the pancaspase inhibitor zVAD-fmk, apoptosis induction by either agent or the combination of both drugs was inhibited, indicating the enhanced apoptosis induction is caspase-dependent (Figure 4B).

Western blot analysis, as shown in Figure 4C, shows enhanced processing of caspase-8 in H460 cells treated with rhTRAIL and bortezomib compared to those treated with rhTRAIL only. The level of c-FLIP, a known inhibitor of caspase-8, was not affected by bortezomib treatment. As bortezomib was reported to increase caspase-8 activation in rhTRAIL-exposed human bladder and prostate cells via a p21-dependent mechanism we evaluated if bortezomib induces p21 in H460 cells [Lashinger et al., 2005]. As shown in figure 4D, p21, as well as p53, is stabilized upon bortezomib treatment, which may also in these NSCLC cells contribute to the observed increased caspase-8 activation upon combination treatment with bortezomib and rhTRAIL.

Additionally it was shown that bortezomib is more potent than rhTRAIL in inducing cleavage of caspase-9. Combining both drugs, enhanced caspase-9 cleavage and diminishing of pro-caspase-9 was observed, resulting in p35 and p37 fragments. Enhanced processing of caspase-9 coincided with rhTRAIL-induced Bid cleavage and bortezomib-induced accumulation of proapoptotic BH3-only protein Noxa. The level of X-linked Inhibitor of Apoptosis (XIAP), a known inhibitor of caspase-3 and caspase-9, was not affected by treatment with bortezomib (Figure 4D). Taken together, the synergistic activity between bortezomib and rhTRAIL occurs both at the level of caspase-8 activation and

mitochondria-dependent caspase-9 activation, resulting in enhanced effector-caspase-3 processing and PARP cleavage.

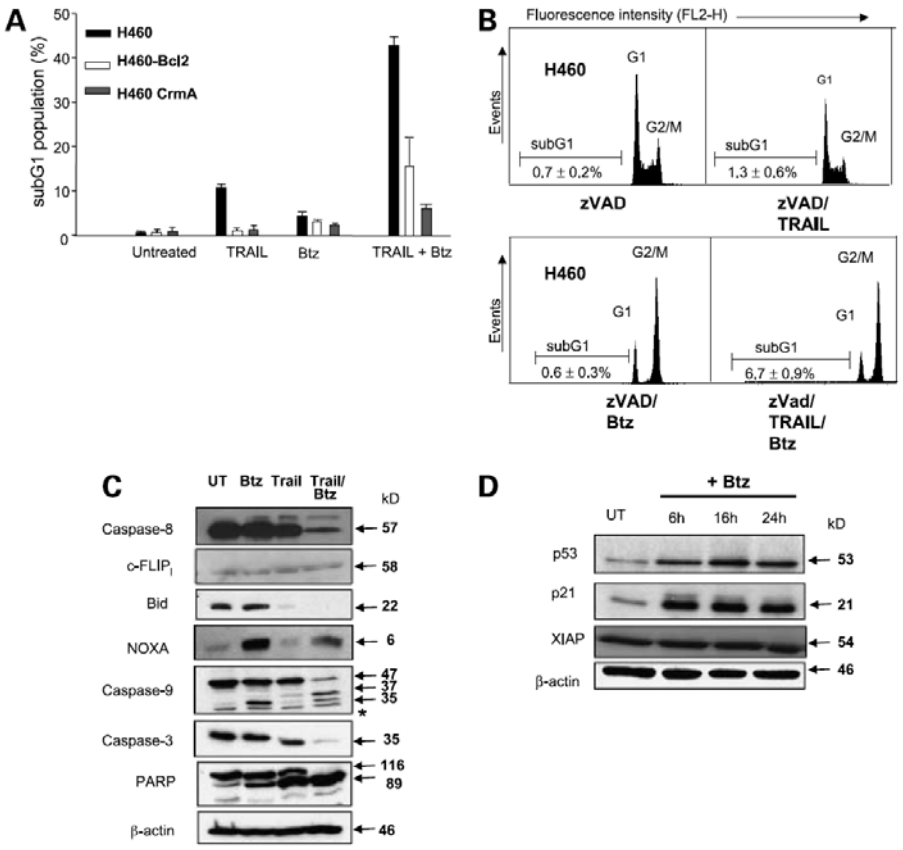
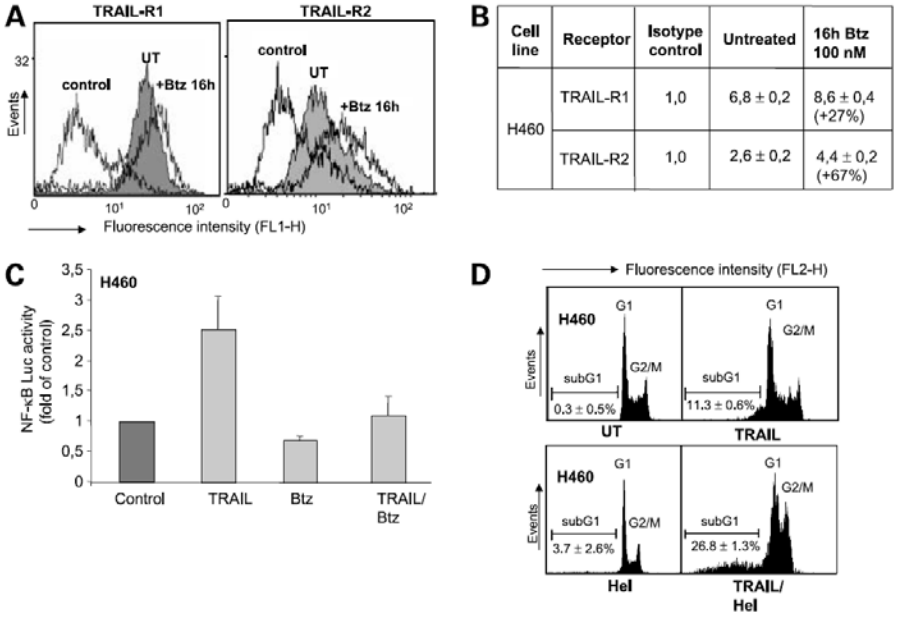


Figure 4. Characterization of apoptosis induction by rhTRAIL and bortezomib in H460 cells.
A, measurement by PI-staining-based FACS analysis of the subG$_1$ fraction of H460 cells and H460 cells stably overexpressing Bcl-2 (H460-Bcl-2) or CrmA (H460-CrmA), upon exposure to bortezomib (100nM), rhTRAIL (50ng/ml) or the combination of both drugs, for 24h. **B,** PI-staining profiles and subG$_1$ fractions (% of total population indicated ± SD) are shown for H460 cells pre-treated with the pancaspase inhibitor zVAD-fmk for 2h and subsequently treated with either rhTRAIL (50ng/ml), bortezomib (100nM), or the combination of both drugs. Means of triplicate determinations ± SD. **C,** expression levels of caspases 8, 9 and 3, as well as PARP, Bid, c-FLIP and NOXA were determined by Western blot analysis of total cell extracts of H460 cells treated with rhTRAIL (50ng/ml), bortezomib (btz) (100nM), or the combination of both drugs, for 24h (*: aspecific band). **D,** expression levels of p53, p21 and XIAP determined by Western blot analysis, in total cell extracts of H460 cells treated with bortezomib 100nM for various periods of time.

Bortezomib induces increased surface expression of death receptors and inhibits rhTRAIL-induced NF-κB activation

We next evaluated if treatment with bortezomib induced the surface expression of the death receptors, to provide a possible mechanism for the enhanced caspase-8 processing in the combination with rhTRAIL. As shown in Figure 5A-B, upon treatment with bortezomib levels of both receptors were increased. This effect was superior for the TRAIL-R2 receptor compared to the TRAIL-R1 receptor. Exposure to bortezomib exceeding 16 hours did not lead to a significant further increase in the level of surface expression of both death receptors (data not shown). Furthermore, rhTRAIL was examined for NF-κB activation potential and the inhibitory effect of bortezomib on NF-κB activation was determined in H460 cells transfected with the NF-κB luciferase reporter construct.



Cell line	Receptor	Isotype control	Untreated	16h Btz 100 nM
H460	TRAIL-R1	1,0	6,8 ± 0,2	8,6 ± 0,4 (+27%)
	TRAIL-R2	1,0	2,6 ± 0,2	4,4 ± 0,2 (+67%)

Figure 5. Mechanisms of sensitization to TRAIL-induced apoptosis by bortezomib in H460 cells. A, levels of constitutive surface expression of TRAIL-R1 and TRAIL-R2 in untreated (UT) H460 cells and upon 16 hours of exposure to bortezomib (100nM), analyzed by FACS using TRAIL-R1 and -R2 antibodies. Isotype-matched antibodies were used to control for unspecific binding. **B,** relative surface expression levels of TRAIL-R1 and TRAIL-R2 compared to the negative control (set at 1.0) in untreated H460 cells and H460 cells treated for 16h with bortezomib 100nM. **C,** effect of rhTRAIL on NF-κB reporter activity in H460 cells. H460 cells were transfected with an NF-κB luciferase plasmid. Transfected cells were cultured with either TRAIL (50 ng/ml), bortezomib (100nM), or in combinations, for 13 hours, at which time luciferase activity was measured. **D,** PI staining profile and the subG$_1$ fraction (% of total population indicated ± SD) of H460 cells treated with Helenalin (10uM), TRAIL (50 ng/ml), or the combination. Means of triplicate determinations ± SD.

Upon treatment with rhTRAIL we observed an approximately 2.5-fold increase in NF-κB activity, which could be effectively reduced to the constitutive level by co-incubation with bortezomib (Figure 5C). These findings thus indicate that part of the apoptosis promoting effects of bortezomib is mediated by inhibition of TRAIL-induced NF-κB activation. Indeed, simultaneous exposure to rhTRAIL (50ng/ml) and Helenalin (10μM), a specific inhibitor of NF-κB, which irreversibly alkylates the p65, resulted in enhanced apoptosis induction compared to rhTRAIL or Helenalin treatment alone (Figure 5D). Nevertheless, apoptosis induction by this combination was significantly less compared to the combination of rhTRAIL and bortezomib, 2.5-fold compared to 4-fold, respectively (see also Figure 3C). As a positive control we tested the effect of bortezomib and Helenalin against TNF-α-induced NF-κB activation, showing comparable capacity at the tested concentrations to inhibit NF-κB activity (data not shown).

Discussion

In the first part of this study we evaluated the cytotoxic effects of TRAIL therapy in NSCLC cells. H460, H292 and H1299 cells were more sensitive to TRAIL-R1 or -R2 antibody-induced growth inhibition than A549 and SW1573 cells. We observed a similar sensitivity profile using AdTRAIL or rhTRAIL. This suggests that activation of either one of the TRAIL receptors is sufficient for the full activation of the apoptotic machinery of the cell.

Increased surface expression of DR4 and DR5 has been associated with TRAIL-sensitivity in for example pancreatic cancer cells, however was not a determinant for TRAIL-sensitivity in lymphoma cells [Georgakis et al., 2005; Khanbolooki et al., 2006]. In NSCLC cells, sensitivity to TRAIL therapy was not associated with the expression levels of the different TRAIL receptors. TRAIL-receptor mRNA transcript levels were comparable among cell lines. Sensitivity could also not be related to the surface expression levels of the receptors. Cells with similar expression levels of TRAIL R1 and R2, e.g. H1299, H292 and SW1573 cells, can either be sensitive or resistant to TRAIL-induced cytotoxicity. Furthermore, the RT-PCR results show higher transcript levels for R1 than R2 in A549 cells but FACS analysis of surface expressed receptors revealed a higher expression of R2 in the cell surface, indicating no direct correlation between transcript and protein levels. This may be caused by a

deficient transport of the TRAIL-receptor to the cell surface, as was recently demonstrated for R1 [Ferreira et al., 2000; Jin et al., 2004].

Alternative mechanisms of resistance could be related to genetic alterations, which are rare and therefore not examined. For example, no mutations in TRAIL-receptors were found in a study evaluating 100 primary NSCLC tumors from white American patients [Wu et al., 2000]. Furthermore, there is also little evidence supporting a high degree of epigenetic silencing by overmethylation of the TRAIL-R1 and TRAIL-R2 receptors in NSCLC. Nevertheless, a 31% rate of aberrant methylation of the so-called decoy receptors, DcR1 or DcR2 was shown [Shivapurkar et al., 2004]. According to our results the NSCLC cell lines studied do not express decoy receptors DcR1 and DcR2 on the cell surface, even though, for the DcR2 receptor, high mRNA transcription levels were detectable in H292, A549 and SW1573 cells. Lacking surface expression of these receptors might contribute to their susceptibility to TRAIL-induced apoptosis compared to normal cells [Kim et al., 2000]. Together, it appears that signalling downstream of receptor activation is the major determinant of sensitivity. This notion is also emphasized by our results obtained with TRAIL/ bortezomib combination therapy. When combining rhTRAIL with bortezomib, both at clinically relevant concentrations, we observed a synergistic enhancement of apoptosis-induction, which led to a similar level of apoptosis-induction in TRAIL-resistant as well as TRAIL-sensitive NSCLC cell lines. Additionally, we showed for the first time that the combination of an adenovirus encoding full length TRAIL and bortezomib also lead to synergistic cytotoxicity. As shown in Figure 6, the synergistic effect by the combination of rhTRAIL and bortezomib is most likely mediated through bortezomib-induced up regulation of the surface expression level of the TRAIL-R2 receptor as well as up regulation of the TRAIL-R1 receptor, resulting in increased caspase-8 activation upon simultaneous treatment with rhTRAIL. This was further indicated by the fact that overexpression of caspase-8 inhibitor CrmA completely abrogated synergistic apoptosis induction. In agreement with our findings, bortezomib has been shown to prevent the degradation of TRAIL-R1 and –R2 in other tumor cell types as well [Johnson et al, 2003]. In contrast to other tumor types, we did not observe a bortezomib-induced decrease in the expression level of caspase-8 inhibitor c-FLIP in H460 NSCLC cells [Conticello et al., 2007]. Others equally showed stabilisation of c-

FLIP in A549 cells upon treatment with proteasome inhibitor MG132 [Liu et al, 2006].

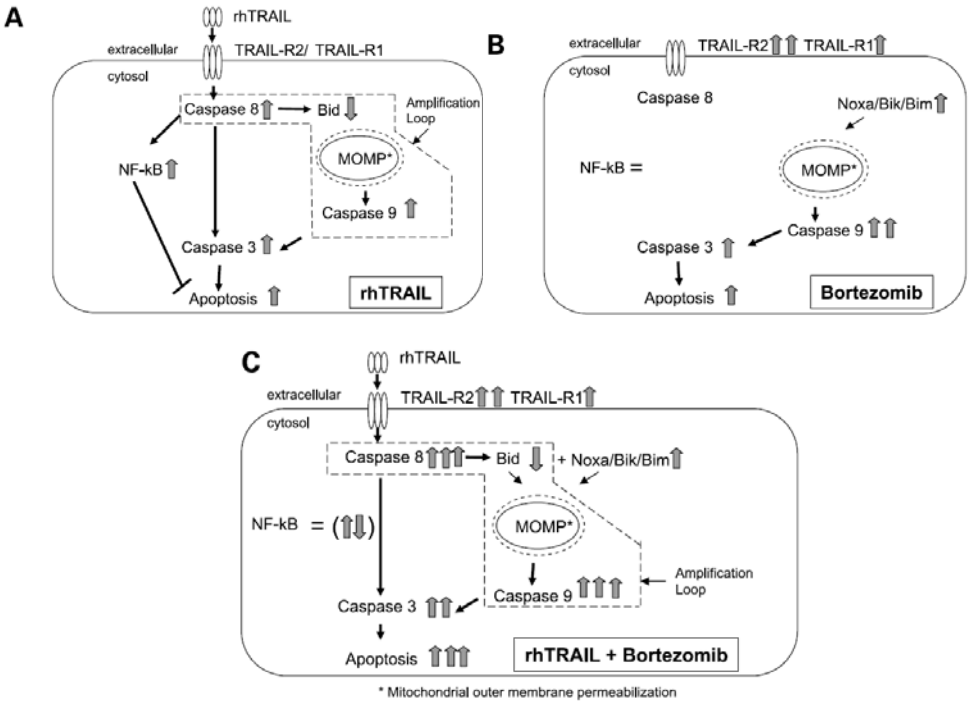


Figure 6. Proposed model for apoptosis activation by rhTRAIL, bortezomib and the combination of both drugs. A, rhTRAIL-induced apoptosis through the death receptor mediated route, including activation of initiator caspase 8 and effector caspase 3, Bid cleavage and subsequent low level activation of caspase 9 ("amplification loop"). TRAIL-induced NF-κB activation suppresses apoptosis. **B,** bortezomib-induced apoptosis through the mitochondria mediated route, including modulation of the balance between Bcl-2 family proteins towards apoptosis (including accumulation of NOXA, Bik and Bim), resulting in MOMP, activation of initiator caspase-9 and effector caspase-3. Bortezomib treatment induces surface expression of TRAIL-R1 and –R2. Bortezomib treatment does not significantly inhibit constitutive NF-kB activation ("="). **C,** synergistic apoptosis activation by combining rhTRAIL and bortezomib results from increased caspase-8 activation, due to increased death receptor surface expression, and increased caspase-9 activation through the amplification loop. Furthermore rhTRAIL-induced NF-κB activation is inhibited by bortezomib co-treatment, resulting in a level equal to untreated cells ("=").

TRAIL-induced apoptosis activation leads to cross-talk between the extrinsic and intrinsic apoptotic pathways via Bid cleavage [Hengartner 2000; Suliman et al., 2001; Abou El Hassan et al., 2004]. This so-called "amplification loop" encompasses activation of the intrinsic or mitochondrial apoptotic pathway as a result of activation of the extrinsic apoptotic pathway. Though Type 2 cells,

treatment with rhTRAIL is likely to result only in a low level of caspase-9 activation, as caspase-9 processing upon rhTRAIL treatment was limited. Others equally showed in NSCLC cells that apoptosis-induction by various TRAIL preparations could only be marginally inhibited by a caspase-9 inhibitor [Kim et al., 2004]. Interestingly, we observed a sensitization to rhTRAIL-induced caspase-9 processing, as caspase-9 processing was superior when the two drugs were combined compared to either treatment alone. This likely results from a combined modulation towards apoptosis of Bcl-2 family members, favoring MOMP and apoptosome mediated caspase-9 processing/ activation. To this effect we observed rhTRAIL-induced processing of proapoptotic BH3-only protein Bid, activating the amplification route, combined with bortezomib-induced accumulation of proapoptotic BH3-only proteins Bik, Bim, and most importantly Noxa [Voortman et al., 2007]. Interestingly, it was shown by others as well that when a (proapoptotic) BH3-mimic agent was combined with rhTRAIL, the combination induces a strong enhancement of apoptosis compared to either agent alone [Yeow et al., 2006]. Notably, we observed that rhTRAIL- as well as bortezomib-induced apoptosis can be inhibited by Bcl-2 overexpression. Bcl-2 is typically an inhibitor of mitochondrial apoptosis, but it was also shown previously by us and others that its overexpression is capable of inhibiting AdTRAIL or rhTRAIL-induced caspase-8 activation as well (Fulda et al., 2002; Abou El Hassan et al., 2004].

Finally, we showed that bortezomib effectively inhibits rhTRAIL-induced NF-κB activation, which might also be a contributing factor to TRAIL therapy-resistance. It has been previously reported by others as well that inhibition of NF-κB activation, e.g. by a p65-specific siRNA construct or an IκB kinase (IKK) inhibitor, preventing nuclear localization of the p65 subunit of NF-κB, could sensitize cancer cells to TRAIL-induced apoptosis [Khanbolooki et al., 2006]. However, in Hodgkin disease cell lines apoptosis induction by bortezomib was independent of inhibitor of NF-kB mutations and synergistic apoptosis induction combining bortezomib with rhTRAIL or agonistic TRAIL-receptor antibodies was hypothesized to result from alternative mechanisms, e.g. downregulation of cFLIP levels [Zheng et al., 2004].

In conclusion, NSCLC cells are differentially sensitive to TRAIL therapy, with no correlation between sensitivity and expression levels of TRAIL-receptors. Treatment with bortezomib sensitizes NSCLC cells to rhTRAIL by increased activation of caspase-8 as well as caspase-9 mediated apoptosis implicating

both the extrinsic and intrinsic route of apoptosis. Combination treatment of bortezomib and rhTRAIL potentially constitutes a new treatment strategy for non-small cell lung cancer patients.

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