



**Bortezomib, but not cisplatin, induces
mitochondria-dependent apoptosis accompanied
by up-regulation of BH3-only protein noxa and
activation of caspase-9 in the non-small cell lung
cancer cell line NCI-H460**

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Abstract

Defects in the apoptotic machinery may contribute to chemoresistance of non-small cell lung cancer (NSCLC) cells. We have previously demonstrated a deficiency in mitochondria-dependent caspase-9 activation in NSCLC H460 cells after exposure to cisplatin, a drug widely used to treat NSCLC. Here we show that, unlike cisplatin, the novel anticancer agent bortezomib efficiently induces caspase-9 activation and apoptosis in H460 cells. A comparative analysis of molecular events underlying cell death in bortezomib- versus cisplatin-treated H460 cells revealed that bortezomib, but not cisplatin, caused a rapid and abundant release of cytochrome c and Smac/DIABLO from mitochondria. This was associated with a marked increase in levels of the BH3-only proapoptotic protein Noxa and the antiapoptotic protein Mcl-1. Taken together, our data show that bortezomib, by promoting a proapoptotic shift in the levels of proteins involved in mitochondrial outer-membrane permeabilization, is a potent activator of the mitochondrial pathway of apoptosis in NSCLC cells. Our preclinical results support further investigation of bortezomib-based therapies as a possible new treatment modality for NSCLC.

Introduction

Apoptosis is an evolutionarily conserved and genetically regulated cell death mechanism playing an essential role in development and maintenance of tissue homeostasis [Strasser et al., 2000]. Many anticancer agents are thought to induce tumor cell death via apoptosis [Gerl and Vaux, 2005]. Defects in the process of apoptosis due to overexpression of antiapoptotic proteins, and/or loss of proapoptotic proteins are frequent in cancer cells, and apoptosis resistance is thought to contribute to their malignant and chemoresistant phenotype [Makin and Dive, 2001; Hanahan and Weinberg, 2000; Shivapurkar et al., 2003].

Pro- and antiapoptotic members of the Bcl-2 protein family are key regulators of the mitochondrial apoptotic pathway. Antiapoptotic members, such as Bcl-2, Bcl-xL and Mcl-1, contain multiple Bcl-2 homology (BH) domains. Proapoptotic Bcl-2 family members, on the other hand, can be subdivided into two classes according to the number of BH domains they bear: multidomain proteins, such as BAK and BAX, or BH3-only proteins, such as Noxa, PUMA, BID, BIM, BAD, BIK and Hrk [Gross et al., 1999; Breckenridge and Xue, 2004]. BH3-only proteins can be further subdivided in activator (BID, BIM) and sensitizer/ de-repressor (Noxa, BIK, BAD, Bmf, Hrk) members. Sensitizer/ de-repressor BH3-only proteins can interact via their BH3 domain with antiapoptotic members, ultimately leading to the activation of BAK and BAX. Activator BH3-only proteins can directly induce oligomerization and activation of BAX or BAK [Letai, 2005]. Activated BAX/BAK, in turn, leads to mitochondrial outer membrane permeabilization (MOMP), causing the release of cytochrome c and Smac/DIABLO, which triggers apoptosis by inducing apoptosome-dependent caspase-9 activation and neutralization of inhibitor of apoptosis proteins (IAPs), respectively [Riedel and Shi, 2004; Green, 2006]. Thus, the balanced expression and the interactions between pro- and antiapoptotic Bcl-2 proteins are critical determinants for a cell to undergo apoptosis or not.

Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancer cases [Bunn et al., 1998]. Platinum-based palliative chemotherapy was long regarded as the standard treatment for advanced NSCLC [Pfister et al., 2004; De Petris et al., 2006]. However, only a minority of patients benefit from this toxic therapy. Therefore, more effective therapies are required [Socinski, 2004]. In this context, a novel approach to cancer treatment is the

use of targeted agents modulating the ubiquitin-proteasome proteolytic pathway, such as bortezomib.

Bortezomib (VELCADE; Millennium Pharmaceuticals Inc, Cambridge, MA and Johnson & Johnson Pharmaceutical Research and Development, LLC, Raritan, NJ) is a dipeptide boronic acid compound that reversibly inhibits the chymotryptic-like proteolytic activity of the 20S proteasome [Lightcap et al., 2000]. Bortezomib has been approved for the treatment of relapsed multiple myeloma and has shown promising antitumor activity, alone or in combination with other cytotoxic drugs, in patients with NSCLC [Bross et al., 2004; Scagliotti, 2006; Aghajanian et al., 2002; Voortman et al., 2005].

Although bortezomib-induced NF- κ B inhibition was shown to be pivotal for the observed anticancer activity in multiple myeloma, other mechanisms might be more important for the observed cytotoxicity induced in solid tumor types such as NSCLC [Hideshima et al., 2001]. In this regard, recent reports indicate that bortezomib treatment induces a proapoptotic shift of the balance between levels of Bcl-2 family member proteins. Proteasome inhibition by bortezomib was shown to induce cleavage of Bcl-2 and accumulation or up-regulation of proapoptotic members of the Bcl-2 family including the BH3-only proteins BIK, BIM and Noxa [Fernandez et al., 2005; Qin et al., 2005; Zhu et al., 2005; Perez-Galan et al., 2006].

We have previously shown that cisplatin-induced activation of caspase-9 is defective in NSCLC H460 cells, and more recent work suggested the presence of a deficiency upstream of the process of apoptosome formation [Ferreira et al., 2000; Checinska et al., 2006]. Here we demonstrate that bortezomib, in contrast to cisplatin, efficiently induces activation of caspase-9 and apoptosis in H460 cells, which coincides with a differential effect of both drugs on the levels of Bcl-2 family proteins involved in MOMP. These findings show that bortezomib can overcome the observed resistance to mitochondria-dependent apoptosis in H460 cells seen after exposure to cisplatin. Consequently, bortezomib may be of therapeutic benefit in treating patients with NSCLC.

Material and methods

Cell culture and drugs

Human NSCLC NCI-H322 (H322) and NCI-H460 (H460) cells were grown in RPMI medium (Cambrex Bioscience, Verviers, Belgium) supplemented with 10% FCS (Life Technologies, Breda, The Netherlands), 100 units/ml penicillin, and 100 μ g/ml

streptomycin (Gibco BRL, Invitrogen, Scotland, UK). We have previously, generated stably transfected derivatives of H460 cells, overexpressing Bcl-2 (H460-Bcl-2) [Ferreira et al., 2000]. H460-Bcl-2 cells were grown in RPMI medium supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin.

Bortezomib (VELCADE) (formerly PS-341) was kindly provided as a pure substance by Millennium Pharmaceuticals Inc. (Cambridge, MA) and dissolved in DMSO. The stock solution of cisplatin (Bristol-Meyers Squibb, Woerden, The Netherlands) was prepared in phosphate buffered saline (PBS).

Growth inhibition assay

A 100µl suspension of 5×10^3 cells was added to each well of 96-well, flat-bottomed plates (Costar, Corning, NY). After 24 h, various concentrations of bortezomib were added to the cells. After 72 h of incubation, growth inhibition was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co., St. Louis, MO) as described previously [Ferreira et al., 2000]. Absorbance values, measured at 540 nm, were expressed as a percentage of untreated controls. The concentrations resulting in cell growth-inhibition of 50% (IC₅₀) or 80% (IC₈₀) were calculated.

Proteasome inhibition assay

To determine the ability of bortezomib to inhibit intracellular proteasome activity of H460 cells, the chymotryptic activity of the proteasome was estimated as described previously [Adrain et al., 2001, Fernandez et al., 2006]. Three $\times 10^5$ H460 cells were incubated for 24 h in the presence or absence of 10nM or 100nM bortezomib. Cells were collected by trypsinization and washed twice in phosphate-buffered saline (PBS). Cell pellets were incubated with 100 µl of lysis buffer (50 mM HEPES, 5 mM CHAPS, 0.5 mM EDTA, 0.035% SDS, pH 7.5) for 1 h on ice. Samples were then centrifuged at 14,000 rpm for 10 min, and supernatants were isolated. Protein (40 µg) was added to 90 µl of lysis buffer, plates were warmed for 10 min at 37°C, and 10 µl of the succinyl-Leu-Leu-Val-Tyr-AMC substrate (BACHEM, King of Prussia, PA) was added to a final concentration of 150 µM. The resultant fluorescence of the released 7-amido-4-methylcoumarin (AMC) dye was measured on a Spectra Fluor multiwell plate reader (Tecan, Salzburg, Austria) set at an excitation wavelength of 380 nm and emission wavelength of 460 nm.

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-Mcl-1, anti-BAX, anti-BAK, anti-BIK (Cell Signaling Technology, Beverly, MA), anti-Bcl-xL/S (Santa Cruz, Santa Cruz, CA), anti-BIM (Axxora, Life Science INC, San Diego, CA) and anti-Smac (Imgenex, San Diego, CA). Mouse monoclonal primary antibodies used were: anti-Bcl-2 (Dako Norden, Glostrup, Denmark), anti-Mcl-1 (clone 22, BD Pharmingen, San

Diego, CA), anti-cytochrome c (BD Pharmingen, San Diego, CA), anti-Noxa (Calbiochem, San Diego, CA), and anti- β -actin (Sigma- Aldrich, Saint Louis, MI). As secondary reagents, horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antibodies (Amersham, Braunschweig, Germany) were used.

Protein interaction assay

For immunoprecipitation experiments, 1×10^8 cells were washed in ice-cold PBS and rinsed with ice-cold lysis buffer (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) supplemented with 1x Protease Inhibitor Cocktail (PIC, Roche Diagnostics GmbH, Mannheim, Germany), 250 μ M PMSF and 1 mM Na_3VO_4 . Cells were scraped, pelleted, resuspended in lysis buffer and incubated for 30 min on ice prior to homogenization with 40 strokes in a 2 ml Dounce homogenizer, using pestle B. After centrifugation at 13.200 rpm for 30 min at 4°C, the supernatants were taken as total cell extracts. Protein concentrations were determined with the Protein Assay Dye Reagent Solution (Bio-Rad, Veenendaal, The Netherlands). Extracts were incubated on ice for 15 min and centrifuged for 5 min at 13200 rpm to remove insoluble particles. Next, extracts were supplemented with 0,1% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS) and 50 mM NaCl, prior to immunoprecipitation with anti-Noxa mAb or anti-Mcl-1 mAb and purification with protein A/G sepharose beads (Santa Cruz, Santa Cruz, CA) O/N at 4°C. Subsequently, beads were washed 5 times in ice-cold lysis buffer, resuspended in loading buffer and the immunoprecipitated proteins were subjected to Western blot analysis.

Subcellular fractionation

Cytosolic and mitochondrial fractions were generated using a previously described digitonin-based subcellular fractionation technique [Waterhouse et al., 2003]. In brief, cells were harvested, washed in ice-cold PBS pH 7.2, and resuspended at a density of 3×10^7 / ml in cytosolic extraction buffer (250 mM sucrose, 70 mM KCl, 137 mM NaCl, 4.3 mM Na_2HPO_4), supplemented with 1x Protease Inhibitor Cocktail, 250 μ M PMSF, and 200 μ g/ml digitonin. After incubation for 5 min on ice, cell membrane permeabilization of at least 95% of the cells was confirmed by staining with 0.2% trypan blue solution. After centrifugation at 3000 rpm for 5 min at 4°C, the cytosolic fraction was recovered. Pellet was solubilized in an equal volume of mitochondrial lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2 % Triton X-100, 0.3% NP-40), supplemented with 1x Protease Inhibitor Cocktail and 250 μ M PMSF. Mitochondrial extracts were recovered by centrifugation at 3000 rpm for 10 min at 4°C. For the detection of cytochrome c and Smac/DIABLO, 20 μ g of cytosolic and mitochondrial fractions were subjected to SDS-PAGE and Western blot analysis.

Flow cytometric analysis of cytochrome c release

Cells (1×10^5) were harvested and treated with 100 μ l digitonin (50 μ g/ml in PBS containing 100 mM KCl and 1mM EDTA) for 5 min on ice until more than 95% were permeabilized as assessed by trypan blue exclusion. Cells were fixed in 3.7% formaldehyde in PBS for 20 min at room temperature, washed three times in PBS and incubated in blocking buffer (3% BSA, 0.05% saponin in PBS) for 1 hour. The cells were incubated overnight at 4°C with anti-cytochrome c monoclonal antibody (BD Pharmingen, San Diego, CA, USA) diluted 1:200 in blocking buffer, washed three times and incubated for 1 hour at room temperature with FITC-conjugated goat anti-mouse (Santa Cruz Biotechnology, St Cruz, CA) diluted 1:200 in blocking buffer. The cells were then analyzed by flow cytometry as described [Ling et al., 2003].

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-G₁) DNA content, was taken as the apoptotic cell population. The percentage of apoptosis indicated was corrected for background sub-G₁ levels found in the corresponding untreated controls.

Caspase activity assay

Caspase-9 and -3 activity was determined by measuring cleavage of the fluorogenic substrates LEHD-AFC or DEVD-AFC, respectively, using commercially available kits (MBL, Co., Nagoya, Japan, BD Biosciences Clontech, Mountain View, CA), according to manufacturer's protocols. Fluorescence was detected using a fluorometer equipped with a 400 nm excitation filter and a 505 nm emission filter (Spectra Fluor Tecan, Salzburg, Austria). Relative caspase activity was determined comparing the level of substrate cleavage in the treated samples vs. untreated controls.

Statistical analysis

When applicable the data were analyzed by the Student's t test. All p-values were considered significant when $p \leq 0.05$. Statistical analysis was performed using the SPSS software program 9.0 (SPSS, Chicago, III).

Results

Bortezomib induces apoptosis in H460 and H322 NSCLC cells more efficiently than cisplatin

H460 and H322 cells were exposed to various concentrations of bortezomib. Figure 1A shows the growth-inhibition curves for the two cell lines. The IC₅₀ values of bortezomib were 49 and 69nM for H322 and H460 cells, respectively.

The IC₈₀ values were 1 μ M and 100nM for H322 and H460 cells, respectively. The IC₅₀ and IC₈₀ concentrations of cisplatin are 2 μ M and 7 μ M in H460 cells and 4 μ M and 10 μ M in H322 cells, as reported previously [Ferreira et al., 2000]. To compare the cytotoxic effect of bortezomib and cisplatin, H460 and H322 cells were treated with a concentration range of bortezomib or cisplatin including the IC₈₀ concentrations of each drug. The percentage of apoptotic cells with sub-G₁ DNA content was evaluated at 48 h by propidium iodide (PI) staining-based fluorescence-activated cell sorter (FACS) analysis. As shown in Figure 1B-E, bortezomib was more effective in inducing apoptotic cell death in H460 and H322 cells than cisplatin. Additionally, a potential synergistic induction of apoptosis by combining bortezomib and cisplatin was tested. Cells were simultaneously or sequentially treated with the IC₈₀ concentration of cisplatin and bortezomib. However, this treatment resulted in a sub-G₁ population comparable to treatment with the IC₈₀ concentration of bortezomib alone (data not shown). Finally, we analyzed the ability of different concentrations of bortezomib to inhibit intracellular proteasome activity in H460 cells.

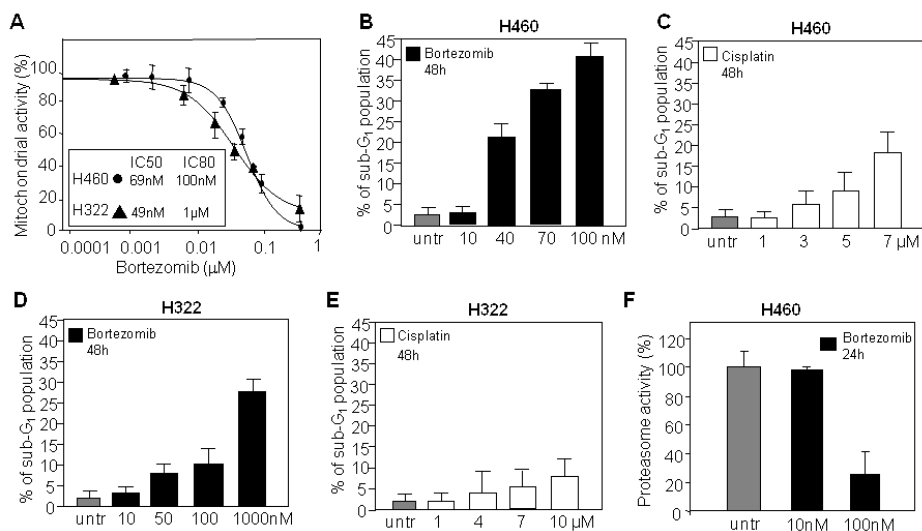


Figure 1. Growth inhibition, induction of apoptosis and proteasome inhibition by bortezomib and cisplatin in NSCLC cells. **A**, growth curves (MTT assays) of H460 and H322 cells treated with different concentration of bortezomib. **B-E**, the sub-G₁ cell fraction was estimated by PI staining-based FACS analysis 48 hours after treatment with a concentration range of bortezomib and cisplatin. Values represent the mean and SD of at least three independent experiments. **F**, intracellular proteasome activity in H460 cells in absence or presence of bortezomib (10nM or 100nM).

As shown in Figure 1F, bortezomib at a concentration of 100nM, but not at 10nM, was able to reduce proteasome activity to approximately 25% of its basal level after 24 h of exposure. Since 100 nM is the IC₈₀ concentration and effectively inhibits proteasome activity in H460 cells, subsequent experiments were carried out using this concentration of bortezomib.

Bortezomib induces strong activation of caspase-9 and caspase-3 activity in H460 cells

The observed superior cytotoxicity of bortezomib in NSCLC cells prompted us to compare the mechanism underlying bortezomib and cisplatin-induced cell death in H460 cells. The processing and activation of caspase-9 and caspase-3 were analyzed in total extracts from H460 cells exposed to IC₈₀ concentrations of either drug. As shown in Figure 2A, processing of procaspase-9 into p35 and p37 cleaved products was already detectable after 24 hours of exposure to bortezomib, further increasing at 48 hours. In contrast, in cells exposed to cisplatin, processing of procaspase-9 was only detected after 48 hours. The predominant cleavage product detected upon cisplatin treatment was a p37 fragment, previously shown to be an inactive form, generated by caspase-3-mediated cleavage of procaspase-9 [Checinska et al., 2006; Zou et al., 2003]. Furthermore, an almost complete processing of procaspase-3 was observed in extracts derived from bortezomib-treated but not cisplatin-treated H460 cells after 48 hours.

Next, LEHD-AFC and DEVD-AFC cleavage assays were used to monitor the activity of caspase-9 and -3 after 24 and 48 hours of treatment with either drug. In line with the processing of procaspase-9 observed by Western blot analysis, treatment with bortezomib for 48 hours induced a 3-fold increase in caspase-9 activity compared to the untreated control ($p < 0.01$) (Figure 2B). At the same time point cisplatin induced no statistically significant increase in caspase-9 activation ($p = 0.06$). Furthermore, bortezomib treatment also induced a more pronounced and rapid increase in caspase-3 activity than cisplatin. As shown in figure 2C, the level of bortezomib-induced caspase-3 activity was approximately 40% higher than the level of cisplatin-induced caspase-3 activity after 48 hours of treatment.

These results demonstrate that bortezomib, in contrast to cisplatin, is a potent inducer of the caspase-9-mediated apoptotic route in H460 NSCLC cells.

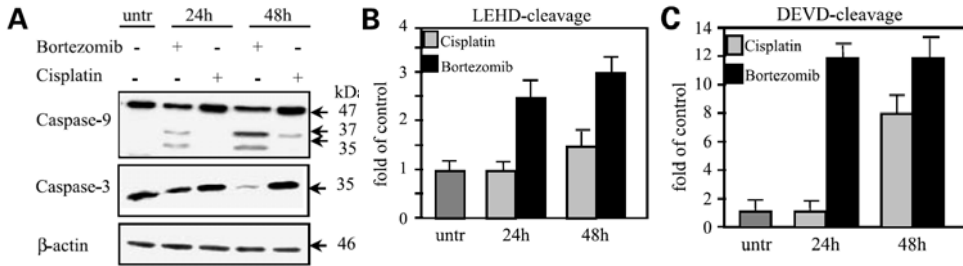


Figure 2. Bortezomib is an efficient inducer of the processing and activation of caspases in H460 cells, when compared to cisplatin.

A, processing of caspase-9 and caspase-3 was determined by Western blot analysis in cytosolic extracts of cells treated with bortezomib (100 nM) and cisplatin (7 μ M) for 24 and 48 hours. **B** and **C**, caspase-9 and caspase-3 protease activity upon 24 and 48 hours exposure to bortezomib or cisplatin, as measured by LEHD-AFC or DEVD-AFC cleavage, respectively. The changes in activity are shown relative to activity in control cells. The mean and SD of at least three independent experiments are shown.

Bortezomib treatment induces cytosolic release of cytochrome c and Smac/DIABLO

We have previously found that the addition of exogenous cytochrome c and dATP to H460 cell extracts induces caspase-9 activation, suggesting that the inability of cisplatin to trigger caspase-9 activation is related to its inability to induce MOMP [Checinska et al., 2006]. Therefore, we hypothesized that the increased cytotoxicity and caspase-9 activation induced by bortezomib could be associated with a more pronounced cytosolic release of cytochrome c. To test this hypothesis, subcellular fractions were generated from H460 cells that were treated with a concentration range of either bortezomib or cisplatin (Figure 3A) or for different periods of time with IC_{80} concentrations (Figure 3B). Western blot analyses showed that cytochrome c release occurred more efficiently after bortezomib treatment than after cisplatin treatment. The amount of cytochrome c released into the cytosol of the cisplatin-exposed cells was only slightly higher than in untreated cells. We quantitated the release of cytochrome c following bortezomib treatment, using flow cytometry [Ling et al., 2003] (Figure 3C). Already after 16 hours of exposure to bortezomib treatment more than 80% of the cells showed reduced fluorescence intensities resulting from diffuse cytoplasmic staining, whereas cisplatin exposure had hardly any detectable effect. Furthermore, we observed that bortezomib, but not cisplatin, promoted the cytosolic release of Smac/DIABLO, which was clearly detectable after 24 hours of exposure to bortezomib (Fig. 3B). These

results show that bortezomib, in contrast to cisplatin, potently induces the cytosolic release of mitochondrial proapoptotic factors in H460 cells.

Differential effects of bortezomib and cisplatin on Bcl-2 proteins

Furthermore, to determine whether the different efficiencies of bortezomib and cisplatin to induce cytosolic release of cytochrome c and Smac is related to differences in their ability to modulate the levels of pro- and antiapoptotic Bcl-2 family members, we compared the effect of both drugs on the expression of Bcl-2 proteins using Western blot analysis. As shown in Figure 4A, we observed an increase in the level of Mcl-1 upon treatment with bortezomib.

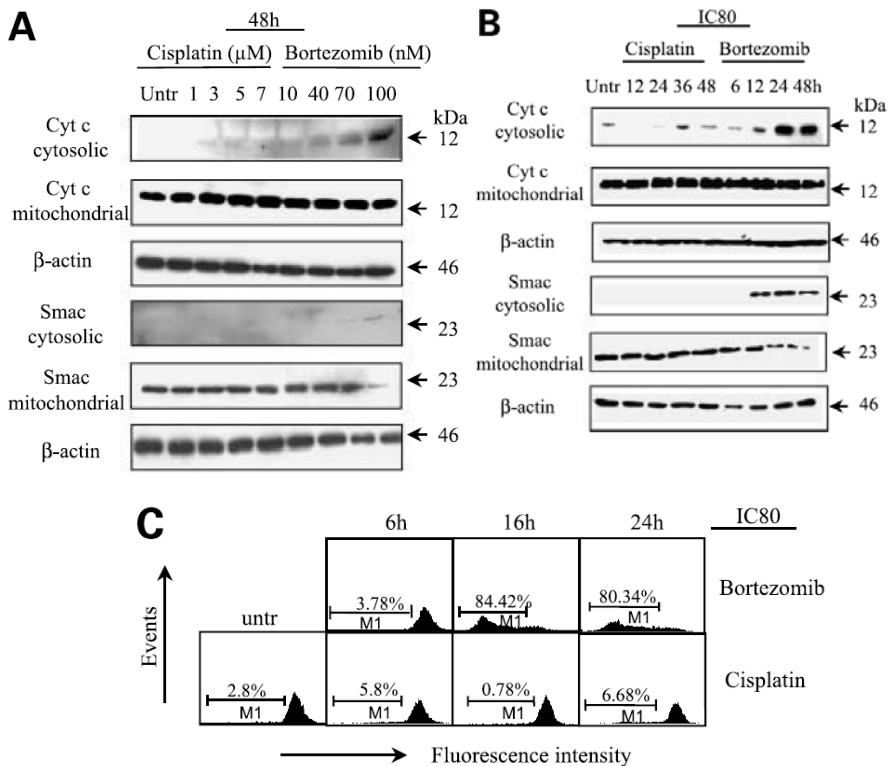


Figure 3. Bortezomib more potently than cisplatin promotes the release of mitochondrial proapoptotic proteins. **A**, cytochrome c and Smac/DIABLO release from mitochondria was determined by Western blot analysis of cytosolic and mitochondrial extracts from a concentration range and a time course treatment of H460 cells with bortezomib or cisplatin. **B**, abundant cytosolic cytochrome c release upon bortezomib treatment, but not cisplatin treatment was confirmed by flow cytometry. **C**, H460 cells were treated with an IC_{80} concentration of either drug for 6, 16 or 24 hours. Loss of cytochrome c from mitochondria resulted in a decrease in fluorescence intensity as determined by flow cytometry analysis. The percentages indicate the proportion of cells with decreased cytochrome c fluorescence intensity as

Upon treatment with cisplatin a slow, time-dependent decrease in the level of Mcl-1 was observed. The levels of Bcl-xL and Bcl-2 were not significantly altered by any of the drugs. Nevertheless the cleavage of Bcl-2 at 48 hours post-treatment was clearly more evident upon bortezomib treatment. The levels of the proapoptotic members BAX and BAK were not affected by treatment with either drug.

The level of proapoptotic BH3-only proteins BIK and BIM were slightly increased upon bortezomib treatment only. Most notably, the BH3-only protein Noxa was dramatically upregulated in cells treated with bortezomib, but not with cisplatin. This was already evident after 6 hours of treatment. These results indicate that bortezomib and cisplatin differentially affect the levels of Bcl-2 family member proteins.

Bortezomib-up-regulated Noxa, displays high affinity for Mcl-1

The differential effects of bortezomib and cisplatin on Bcl-2 family proteins expression may provide an explanation for the potent induction of MOMP by bortezomib in contrast to cisplatin. As shown above, bortezomib increases the expression of both proapoptotic Bcl-2 proteins, BIK, BIM and especially Noxa, and the antiapoptotic Bcl-2 protein Mcl-1, which is sufficient for the triggering of mitochondria-dependent apoptosis as demonstrated by the increased release of mitochondrial proapoptotic factors and the activation of caspases. Although the level of Mcl-1 is gradually reduced in cisplatin treated cells, Noxa remains undetectable even upon prolonged cisplatin exposure.

We used a co-immunoprecipitation approach to examine the interaction between Noxa and Mcl-1 in H460 cells treated with bortezomib. Western blot analysis of total cellular extracts confirmed the accumulation of Mcl-1 upon bortezomib treatment. Subsequent pull-down of Noxa demonstrates the binding of Noxa to Mcl-1 (Figure 4B). Additionally, reverse co-immunoprecipitation of Mcl-1 confirmed the interaction between Noxa and Mcl-1 in bortezomib-treated H460 cells.

This finding suggests that the observed Noxa/Mcl-1 binding in bortezomib-treated cells is able to release proapoptotic Bcl-2 family proteins bound to Mcl-1, such as activator BH3-only proteins or BAK, thereby facilitating MOMP and apoptosis [Letai et al., 2005; Perez-Galan et al., 2006].

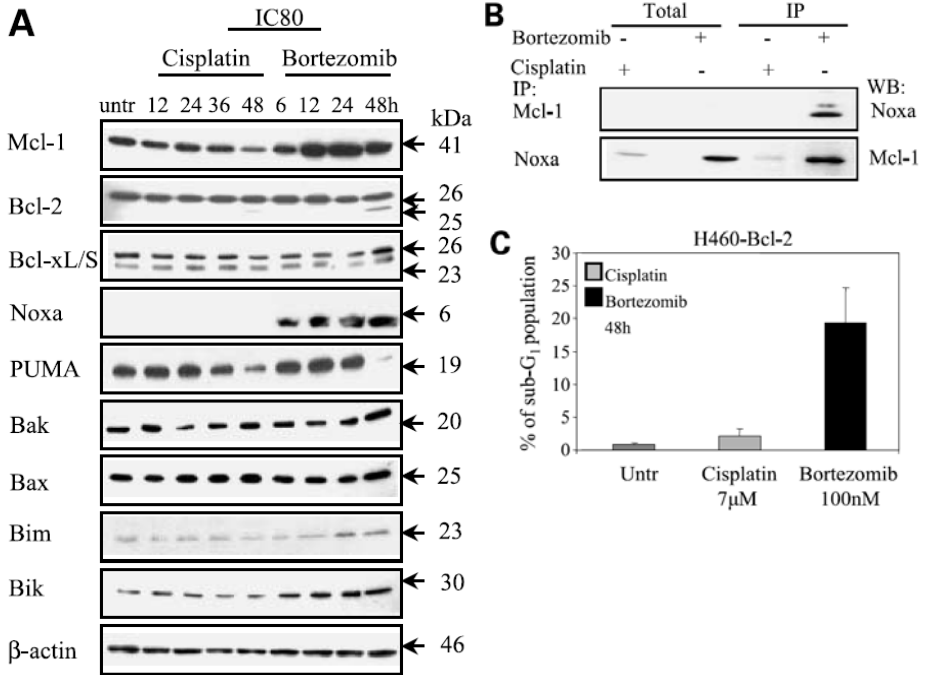


Figure 4. Involvement of the Bcl-2 family in mediating bortezomib-induced apoptosis. Increased levels of Noxa and Mcl-1 proteins after bortezomib treatment. **A**, time course Western blot analysis of total cell extracts of H460 cells showing the effects of treatment with IC₈₀ concentration of cisplatin (7 μM) and bortezomib (100 nM) on the levels of antiapoptotic and proapoptotic Bcl-2 family member proteins **B**, bortezomib-induced binding and interaction of Noxa and Mcl-1 in H460 cells. Noxa or Mcl-1 were pulled down from total cell extracts, untreated or treated with bortezomib (100 nM) for 24 hours. Levels of co-precipitating Mcl-1 or Noxa were analysed using Western blot analysis. Total lysate was included as a control. **C**, bortezomib (100nM), but not cisplatin (7μM), induces apoptosis in H460-derived cells stably overexpressing Bcl-2.

Bortezomib-induced apoptosis is partially inhibited by ectopic overexpression of Bcl-2

Finally, to further study the role of Bcl-2 family members in mediating bortezomib-induced apoptosis H460-derived cells stably overexpressing exogenous Bcl-2 (H460-Bcl-2) were used. As shown in Figure 4C, 100 nM of bortezomib induced significant levels of apoptosis in these cells (approximately 20%) when compared to cisplatin that hardly triggered apoptosis. However, the apoptotic potential of bortezomib was reduced by approximately 50% when compared to the levels obtained in parental H460 cells (approximately

40% of apoptotic cells; Figure 1B). The strong reduction of cisplatin-induced apoptosis in H460 cells by Bcl-2 (Figures 1C and 4C) also suggests the involvement of this pathway in mediating cell death by this drug as we reported before, although the mechanism remains elusive considering the failure of cisplatin to induce MOMP in NSCLC cells [Ferreira et al., 2000].

Discussion

Bortezomib is a potent and selective proteasome inhibitor. It is a new, promising anti-neoplastic therapeutic agent that currently has been approved for the treatment of relapsed multiple myeloma. Bortezomib as a single agent achieved a response rate of 10% in unselected advanced NSCLC patients who had already received one prior chemotherapy regimen [Fanucchi et al., 2005]. Intrinsic apoptosis resistance of NSCLC cells is regarded as one of the prime mechanisms for the observed chemoresistance in this cancer type [Yang et al., 2003]. We, and others, have provided experimental evidence that suppression of mitochondria-dependent caspase-9 activation contributes to the drug resistant phenotype, in particular towards DNA damaging agents (Ferreira et al., 2000; Checinska et al., 2006; Yang et al., 2003; Kruyt et al., 2003, Krepela et al., 2004], although the exact molecular mechanism underlying this inhibition remains to be elucidated. New therapeutic strategies are needed, which can effectively bypass or restore apoptotic defects and possibly improve the poor survival rates of NSCLC patients. Here, we compared the apoptosis-inducing activity of bortezomib to that of cisplatin in H460 and H322 NSCLC cell lines. We then compared the molecular mechanism of apoptosis induction by bortezomib and cisplatin in one of these cell lines, e.g. H460. Bortezomib was able to effectively inhibit proteasome activation in these cells. Interestingly, bortezomib, in contrast to cisplatin, induced rapid activation and processing of caspase-9 and caspase-3 activation, which was followed by a strong apoptotic response. Moreover, bortezomib stimulated a pronounced release of mitochondrial pro-apoptotic factors cytochrome c and Smac/DIABLO, whereas cisplatin did not. These findings are consistent with a previous report implicating the formation of radical oxygen species (ROS) and loss of mitochondrial membrane potential in bortezomib-induced cytotoxicity in NSCLC cells [Ling et al., 2003]. Additionally, we have recently reported that the addition of exogenous cytochrome c and dATP induces efficient apoptosome formation and caspase-9 activation in H460 cell extracts,

indicating no intrinsic defects in this pathway, which is in agreement with the observed potent activation of this route by bortezomib [Checinska et al., 2006]. Taking into account all these observations, we propose that the amount of cytochrome c release upon cisplatin treatment is below the threshold needed to initiate the activation of the intrinsic pathway of apoptosis, whereas the abundant cytochrome c release induced by bortezomib is sufficient to induce caspase-9 activation. Interestingly, bortezomib, but not cisplatin,

Table 1. Molecular events underlying the differential activation of the mitochondrial apoptotic pathway by cisplatin and bortezomib in H460 cells.

	Treatment	
	Cisplatin	Bortezomib
Levels of Bcl-2 proteins		
<i>Proapoptotic</i>		
Noxa	–	+++
BAK	+++	+++
BAX	+++	+++
BIM	+/-	+
Bcl-xS	++	++
BIK	+	++
Bcl-2 (cleaved)	+	++
<i>Antiapoptotic</i>		
Mcl-1	+	+++
Bcl-2	+++	+++
Bcl-xL	++	++
Release of mitochondrial proapoptotic factors		
Cytochrome c	+	+++
Smac/DIABLO	–	++
Activation of caspases		
Caspase-9	–	+++
Caspase-3	++	+++
Cell death	++	+++

Abbreviations: +, weak; ++, moderate; +++, strong; –, absent.

induced the release of the inhibitor of apoptosis proteins (IAPs)-antagonizing protein Smac/DIABLO, which additionally may contribute to the strong apoptotic response induced by bortezomib treatment [Du et al., 2000].

The mitochondrial outer membrane permeabilization (MOMP), which determines the release of cytochrome c into the cytosol, is critically regulated by the balanced expression and interactions between proapoptotic and antiapoptotic members of the Bcl-2 family of proteins [Cory et al., 2003; Lucken-

Ardjomande and Martinou, 2005; Gelinas and White, 2005]. The treatment with bortezomib or cisplatin revealed distinct effects on the expression level of various Bcl-2 family proteins. Bortezomib treatment altered the balance between Bcl-2 proteins towards apoptosis by inducing a dramatic increase in the levels of BH3-only "sensitizer" proteins Noxa and, to a lesser extent, BIK, and increasing the level of BH3-only "activator" protein BIM. Concomitantly, an increase in the level of antiapoptotic protein Mcl-1 was detected.

Interestingly, here we showed that bortezomib-induced apoptosis in NSCLC cells involves the up-regulation of three BH3-only proteins, Noxa, BIM and

BIK, in combination with accumulation of Mcl-1. Similar observations have been made in other cancer types; e.g., bortezomib was reported to increase the expression of NOXA and Mcl-1 in melanoma or mantle-cell lymphoma cells, and of BIK and BIM in different cancer cell lines [Fernandez et al., 2005; Zhu et al., 2005; Qin et al., 2005; Perez-Galan et al., 2006]. In our study, we demonstrated that Noxa and Mcl-1 strongly interact in bortezomib-treated H460 cells. It seems that up-regulated Noxa is able to antagonize the antiapoptotic effect of Mcl-1. This observation is in agreement with a recently published report showing that the increase of Noxa and Mcl-1 in bortezomib-treated mantle-cell lymphoma cells leads to the release of Bak from its inhibitory binding to Mcl-1, thus triggering apoptosis [Perez-Galan et al., 2006]. According to the model that was proposed by Letai et al. (Letai, 2005a; Letai et al., 2005b), up-regulated expression of sensitizer BH3-only proteins Noxa and BIK can force the replacement of activator BH3-only proteins, such as up-regulated BIM, from binding to antiapoptotic proteins, such as Bcl-2, Bcl-xL and Mcl-1. Released BIM can subsequently promote the activation and oligomerization of BAK/BAX and induce MOMP followed by cytochrome c release and induction of cell death.

In line with the above we showed that Bcl-2 overexpression in H460 cells partially suppresses bortezomib-induced apoptosis, further indicating that the Bcl-2 family plays a substantial role in mediating apoptosis by this agent. On the other hand, since Bcl-2 overexpression did not completely inhibit apoptosis also other yet not defined mechanisms are involved in facilitating bortezomib-induced apoptosis in these cells.

Table 1 summarizes the differences that were detected between bortezomib and cisplatin-induced mitochondrial-dependent apoptosis in NSCLC cells. The detailed molecular events underlying the deficiency of cisplatin to efficiently induce cytosolic cytochrome c release are currently under investigation.

This study demonstrates the greater potential of bortezomib, compared to cisplatin, to efficiently activate the mitochondrial pathway of apoptosis in NSCLC H460 cells, by modulation of the balance between Bcl-2 family proteins towards apoptosis. When extrapolated to the clinic, these *in vitro* findings might provide a rationale for the use of bortezomib-based anticancer therapy as a potential treatment strategy for NSCLC.

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