Extensive Immunoglobulin Production Sensitizes Myeloma Cells for Proteasome Inhibition

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Abstract

Multiple myeloma is an incurable plasma cell neoplasia characterized by the production of large amounts of monoclonal immunoglobulins. The proteasome inhibitor bortezomib (PS-341, Velcade) induces apoptosis in various malignant cells and has been approved for treatment of refractory multiple myeloma. Inhibition of the antiapoptotic transcription factor nuclear factor-κB (NF-κB) apparently contributes to the antitumor effects of bortezomib; however, this mechanism cannot fully explain the exceptional sensitivity of myeloma cells. Extensive protein synthesis as in myeloma cells is inherently accompanied by unfolded proteins, including defective ribosomal products (DRiPs), which need to be degraded by the ubiquitin-proteasome system. Therefore, we hypothesized that the proapoptotic effect of bortezomib in multiple myeloma is mainly due to the accumulation of unfolded proteins in cells with high protein biosynthesis. Using the IgG-secreting human myeloma cell line JK-6L and murine μH-chain–transfected Ag8.H myeloma cells, apoptosis induction upon proteasome inhibition was clearly correlated with the amount of immunoglobulin production. Preferentially in immunoglobulin-high myeloma cells, bortezomib triggered activation of caspases and induction of proapoptotic CHOP, a component of the terminal unfolded protein response induced by endoplasmic reticulum (ER) stress. In immunoglobulin-high cells, bortezomib increased the levels of proapoptotic Bax while reducing antiapoptotic Bcl-2. Finally, IgG-DRiPs were detected in proteasome inhibitor–treated cells. Hence, proteasome inhibitors induce apoptosis preferentially in cells with high synthesis rate of immunoglobulin associated with accumulation of unfolded proteins/DRiPs inducing ER stress. These findings further elucidate the antitumor activities of proteasome inhibitors and have important implications for optimizing clinical applications.

Introduction

Multiple myeloma, a malignancy of plasma cells, results in the overproduction of monoclonal immunoglobulins. Although the hitherto existing therapeutic strategies, such as chemotherapy, radiotherapy, autologous, and allogeneic stem cell transplantation, lead to prolonged survival of multiple myeloma patients, these therapies rarely induce long-lasting complete remissions but frequently are associated with severe adverse effects (1). Recently, the dipeptidyl boronic acid derivative bortezomib (PS-341, Velcade), a reversible, highly selective and potent inhibitor of the 26S proteasome, has been approved for treatment of multiple myeloma progressing on prior therapy (2, 3). According to recent studies, bortezomib may also be part of very effective frontline therapy.

The 26S proteasome constitutes a multienzyme complex present in all eukaryotic cells that plays an essential role in degrading supernumerous, defective, or misfolded proteins, which are targeted for proteasomal degradation by ubiquitinylatination. In addition, the proteasomes are involved in the regulation of cellular processes, including cell cycle and apoptosis, by controlling the degradation of key regulatory factors (4). Bortezomib and other proteasome inhibitors induce apoptosis in many different types of cancer cells; however, myeloma cells seem to be exceptionally sensitive (5, 6). The molecular mechanisms of this hypersensitivity have not yet been fully characterized.

In general, it is believed that the antimyeloma effect of bortezomib is, at least partially, due to inhibition of the transcription factor nuclear factor-κB (NF-κB). Constitutive NF-κB activity in myeloma cells mediates survival as well as resistance to chemotherapy and radiotherapy (6, 7), among other effects, by inducing the expression of antiapoptotic proteins, adhesion molecules, and autocrine growth factors such as interleukin-6 (IL-6; ref. 5). NF-κB is normally sequestered in the cytoplasm as an inactive complex with inhibitory proteins called IκBs. Activation of NF-κB requires signal-induced proteasome-dependent degradation of IκBs, thereby releasing NF-κB that then activates transcription of its target genes (8).

However, proteasome inhibitors may have additional effects on myeloma cells beyond the blockade of NF-κB because a specific inhibitor of the IκB kinase β (IKKβ) reduced proliferation of myeloma cells markedly less effectively than bortezomib (7). In consistency, we also observed that myeloma cell lines were markedly more sensitive to bortezomib when compared with other, more specific NF-κB inhibitors.

Proteasome inhibition prevents the clearance of unfolded proteins by the endoplasmic reticulum (ER)–associated degradation pathway (ERAD), resulting in an ER stress response (9, 10). This so-called unfolded protein response (UPR) is a signaling...
pathway from the ER to the nucleus triggered by the accumulation of misfolded proteins in the ER lumen (11, 12). The UPR includes three mechanisms to handle the vast increase of unfolded proteins: transcriptional induction of target genes enhancing protein folding, general translational repression, and ERAD to eliminate misfolded proteins (13). UPR activation seems to be essential for plasma cell differentiation and survival by inducing ER chaperones and folding enzymes. However, overwhelming ER stress activates terminal UPR, leading to apoptosis (14–17).

Based on these findings, we hypothesized that the proapoptotic effect of bortezomib in myeloma cells producing large amounts of secreted immunoglobulin is due to the accumulation of IgG-derived defective ribosomal products (DRiPs) and other unfolded proteins in the ER and cytoplasm. Therefore, the level of ER proteins synthesized in cells should correlate with their sensitivity towards proteasome inhibition. To address this question, we investigated the effects of proteasome inhibitors, such as bortezomib, on the viability of myeloma cell clones producing various amounts of immunoglobulin. We found a clear correlation of the immunoglobulin chain expression level with the sensitivity towards proteasome inhibitors in subclones of the human IgG-secreting myeloma cell line JK-6L and in subclones of the murine myeloma cell line Ag8 transfected with an expression plasmid encoding the μ heavy (H)-chain. Moreover, proapoptotic factors of the ER stress response were much stronger induced in subclones producing high levels of μ-chains than in those producing no μ-chains.

Materials and Methods

Antibodies. The following primary antibodies were used: monoclonal mouse anti-GRP78 (BiRBD Pharmaingen, Heidelberg, Germany), rabbit polyclonal anti-actin (Sigma, Taufkirchen, Germany), rabbit polyclonal anti-caspase-12 (Acros Antibodies GmbH, Hiddernhausen, Germany), rabbit polyclonal anti-XBP-1, mouse monoclonal anti-CHIP, rabbit polyclonal anti-Bcl-2, and mouse monoclonal anti-Bax (all from Santa Cruz Biotechnology, Santa Cruz, CA). As secondary antibodies, the following antibodies were used: horseradish peroxidase–conjugated goat anti-mouse IgG, goat anti-rabbit IgG (Amersham Biosciences, Freiburg, Germany), donkey anti-goat IgG (Santa Cruz Biotechnology), FITC-conjugated goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL), and FITC-conjugated goat anti-human IgG (Caltag, Burlingame, CA).

Reagents. MG-132, lactacystin, and MG-262 were received from Calbiochem (Schwalbach/Ts, Germany); bortezomib (PS-341, Velcade) was obtained from the University Hospital Erlangen; and tunicamycin was purchased from Sigma.

Cell lines and culture conditions. Cell lines used in this study were grown in RPMI 1640 supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, 1 mM/L sodium pyruvate, 2 mM/L L-glutamine, 10% FCS for mouse myeloma cells, and 20% FCS for JK-6L cells. Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. We established the Ag17.2.25 VH region, which is able to pair with immunoglobulin light chains (21). The polyclonally generated sequence of the secreted form was deleted. Stable transfectants were selected in complete RPMI supplemented with 2.5 μg/mL mycophenolic acid, 250 μg/mL xanthine, 100 μM/L hypoxanthine, and 16 μM/L thymidine (HT-media supplement; Sigma-Aldrich, Taufkirchen, Germany). To obtain Igκ-expressing Ag8 cells (Ag8κ), Ag8H cells were transfected with an expression plasmid encoding murine Igκ (22). Single-cell clones from Ag8κH and JK-6L cells were established by limiting dilution.

Flow cytometric analysis. Surface staining was done as described (23). For intracellular staining, cells were fixed and permeabilized using Cycofix/ Cytoperm (BD, Heidelberg, Germany), stained with the appropriate amount of antibodies, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA). Data analyses were done using the CellQuest software (BD Biosciences).

Viability assay. Cells (4 × 10⁴) were treated with different concentrations of proteasome inhibitors for 24 h and incubated for another 4 h with AlamarBlue (15 μL added to 200 μL of culture; BioSource International, Inc., Camarillo, CA). Activity of the mitochondrial dehydrogenase results in conversion of the coloring, which was followed by measurement of the absorption using a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA).

Cell cycle analysis. Cells (1 × 10⁵) were treated with 1 μM/L bortezomib for indicated time intervals. Harvested cells were resuspended in a staining solution containing 0.6 μg/mL propidium iodide (Sigma-Aldrich), 4 mM/L sodium citrate, and 0.1% Triton X-100 and incubated for 24 h at 4°C. DNA content was determined by flow cytometry (Epics XL, Coulter Co., Miami, FL).

Cell death analysis. Cells (1 × 10⁵) were treated with different concentrations of bortezomib for indicated time intervals; 50 μM/L of the broad-range caspase inhibitor zVAD-FMK (Alexis Biochemicals, San Diego, CA) was used to block caspase activation. Staining with Annexin V–FITC (BD-Pharmingen, Erlangen, Germany) and propidium iodide (Sigma-Aldrich) was done as described (24). The fluorescent dye 3,3′-dihexyloxacarbocyanine iodide (DiOC₆; Sigma) was used to reveal disruption of the mitochondrial transmembrane potential (ΔΨₘ). For measurement, 1 × 10⁵ cells were treated with 1 μM/L bortezomib for indicated time intervals. Afterwards, cells were incubated with DiOC₆ dye for 20 min at 37°C and analyzed by flow cytometry.

Caspase-3/7 assay. Cells (5 × 10⁴) were treated with 1 μM/L bortezomib for 16 h. Caspase-3/7 activity was measured with the Caspase-Glo 3/7 assay (Promega, Madison, WI) according to the manufacturer’s instructions. Luminescence was measured using a luminometer (Sirius, Berthold Detection Systems, Pforzheim, Germany).

Immunoblotting. Cells (1 × 10⁴) were treated with 1 μM/L bortezomib for indicated time intervals. Equal amounts of cells were washed with PBS and directly lysed in SDS sample buffer containing β-mercaptoethanol to generate total cell lysates. Proteins were separated on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk (Roth, Karlsruhe, Germany) in TBS solution containing 0.05% Tween 20 (Merck, Hohenbrunn, Germany) for 1 h. Blots were probed with antibodies and developed using the enhanced chemiluminescence method.

Reverse transcription–PCR analyses. Cells (1 × 10⁴) were treated with 1 μM/L bortezomib for indicated time intervals. Total RNA was isolated using the Mini RNAeasy kit (Qiagen, Hilden, Germany). Aliquots of 1 μg total RNA were treated with the Superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) and amplified with Taq polymerase (NEB, Frankfurt/Main, Germany) using specific primers for XBP-1 (forward, CCTTGTGTGTGAGAACCAGG; reverse, TCTCTGCTCTAGAGGGGATGT), and glyceraldehyde-3-phosphate dehydrogenase (forward, AGTCCTAGTGACTTCCCCGTTCAGC; reverse, TGGTATCGTGGAAAGGACTGATG). PCR products were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining. For quantitative real-time PCR analysis, the cDNA and appropriate primers (Bcl-2 forward, ATGGCTTTGGAAGCTATAGTG; Bcl-2 reverse, GGATGACCCACAGTGATG; Bax forward, TGAAGACAGGGGCTTTCCT; Bax reverse, AATTTGCGGACAGCTCT; I-βs forward, TGAAGGACGGAGGTGACC; I-βs reverse, TTCTGTGTGTGAGAACCAGG; β-actin forward, GGCTGTATCCCTTCCATCG; β-actin reverse, CCAGTTGGTAAAATGCCTAGT) were mixed with 2× Absolute qRT-PCR SYBR green ROX reagent (Applied Biosystems, Darmstadt, Germany). Quantitative real-time PCR was done in triplicates in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, Darmstadt, Germany).
Electrophoretic mobility shift analysis. Electrophoretic mobility shift assays (EMSA) were done as described previously (25). Briefly, nuclear extracts were prepared from cells (5 x 10^6) treated with 1 μmol/L bortezomib for indicated time intervals. Ten micrograms of nuclear extracts were added to the probe mix. Probes were end labeled with 32P-dGTP (Hartmann Analytik, Braunschweig, Germany). The reaction mixtures were incubated at room temperature for 15 min and run on a 4% non-denaturing polyacrylamide gel. For specificity control, nuclear extracts were incubated for 15 min at room temperature with unlabeled double-stranded oligonucleotides or a respective mutant variant at 100-fold molar excess. Gels were dried on Whatman paper and exposed to X-ray film for 48 h at -70°C. Alternatively, gels were analyzed using phosphoimaging instrumentation (Fujifilm FLA-3000, Agilent Technologies, Palo Alto, CA).

Metabolic labeling. For detection of IgG DRiPs, short-term pulse-chase experiments were conducted following previously published standard DRiP pulse-chase protocols (26, 27). Briefly, JK-6L cells were treated with 25 μmol/L of each MG-132 and lactacystin or DMSO during 10 min of starvation in methionine-free, serum-free RPMI (Invitrogen, Life Technologies, Karlsruhe, Germany). Cells were radiolabeled for 5 min with 1.5 mCi/mL 35S-methionine (Amersham Life Sciences, GE Healthcare, Munich, Germany) and chased for up to 30 min while shaking at 37°C in Iscove’s DMEM supplemented with 10% FCS and 10 mmol/L methionine in the presence or absence of proteasome inhibitors. Cells were lysed in 200 μL CHAPS-deoxycholate buffer for 5 min on ice and separated from the insoluble fraction by centrifugation at 20,000 x g for 10 min. IgG proteins were recovered from the soluble fraction by immunoprecipitation using anti-human IgG antibodies (mixture of polyclonal rabbit and goat anti-human IgGs from Jackson Immuno Research Laboratories, Inc., West Grove, PA) prebound to Protein G-Sepharose. Samples were separated by SDS-PAGE on a 10% Acrylamide Prosieve gel (FMC Bioproducts, Rockland, ME). Following fixation for 1 h in 40% methanol, 10% acetic acid, gels were rinsed with water, soaked in 1 mol/L sodium salicylic acid solution for 5 h, and dried. Radioactivity in gels was analyzed using phosphorimaging instrumentation (Fujifilm BAS-2000, Fujifilm, Tokyo, Japan) or fluorography.

Results
Sensitivity to bortezomib-induced apoptosis depends on immunoglobulin expression levels. First, we wanted to determine the sensitivity of myeloma cells secreting high versus low amounts of IgG towards proteasome inhibitors (Fig. 1A). For this...
purpose, we tested >500 subclones of the human myeloma cell line JK-6L, which naturally secretes IgG, for the levels of intracellular IgG expression by flow cytometry. The maximal difference between IgG-producing cells was up to 5-fold (Fig. 1B). Selected subclones producing different amounts of IgG were treated with increasing concentrations of proteasome inhibitors, and viability was determined. As shown in Fig. 1C, the viability of a high (JK-6L.31H) and a low (JK-6L.39L) IgG-producing subclone was strongly and dose-dependently reduced by concentrations of bortezomib as low as 10 nmol/L. However, the subclone producing higher levels of IgG was significantly more affected by proteasome inhibition, although the difference in IgG synthesis was at the most 5-fold. Similar results were obtained for other high and low IgG-producing JK-6L subclones (data not shown). The correlation between IgG expression and sensitivity towards proteasome inhibition was also observed using another proteasome inhibitor (MG-262) that reversibly blocks the proteasome by a different mechanism (data not shown).

To further challenge our hypothesis that the level of immunoglobulin biosynthesis determines the sensitivity towards proteasome inhibition, we established an in vitro model, based on the murine myeloma cell line Ag8, which express neither endogenous immunoglobulin heavy (H) nor light (L) chains (Fig. 1A). The Ag8.H cells were transfected with an expression plasmid encoding the membrane form of a μH-chain (Fig. 1A); hence, this μH-chain cannot be secreted. Due to the lack of LgL chains, there is also no surface expression detectable. Expression of μH-chain was analyzed by flow cytometry (Fig. 1B) and Western blot analysis using an anti-IgM antibody (data not shown). The viability of Ag8 cells synthesizing μH-chains (Ag8.H) was drastically reduced with increasing proteasome inhibitor concentrations, whereas the non-transfected parental cells (Ag8.H) seemed virtually unaffected by the proteasome inhibitor (Fig. 1C). In addition, expression of another ER-synthesized protein Igk, which is a signal transducing component of the B-cell receptor complex, rendered Ag8 cells hypersensitive towards bortezomib (Fig. 1C). These results clearly indicate that the sensitivity of myeloma cells towards proteasome inhibitors depends on the synthesis of immunoglobulin chains or other proteins processed within the ER.

To exclude artifacts caused by the transfection and selection procedure, we generated subclones of Ag8H1 cells to obtain clones with and without μH-chain expression (Fig. 1A). In Fig. 1A, flow cytometric analyses of μH-chain expression of two Ag8H1 subclones, one that lost μH-chain expression (Ag8H1-) and one that still expressed the μH-chain (Ag8H1+), are displayed. To exclude the possibility of differences in proteasome function between Ag8μH- and Ag8μH+ subclones, we determined the proteasomal chymotrypsin-like activity. Because proteasomal proteolysis requires the expenditure of ATP, we also investigated whether any alterations exist in respect to cellular ATP contents. Our results showed that neither the proteasomal activity nor the ATP status was significantly different between Ag8μH- and Ag8μH+ cells (data not shown). In addition, Ag8μH- versus Ag8μH− subclones traverse through cell cycle with similar kinetics (Fig. 2A). Interestingly, in the presence of bortezomib, G2 arrest occurs earlier and is more pronounced in Ag8μH+ compared with Ag8μH− cells (Fig. 2A).

The following figures show experiments using the two subclones Ag8μH- (Ag8H1.12) and Ag8μH+ (Ag8H1.11) as shown in Fig. 1A; however, similar results were obtained using other μH-chain-negative as well as μH-chain-positive subclones (data not shown). We investigated by three independent assays whether decreased viability in bortezomib-treated Ag8μH+ compared with Ag8μH− cells is due to induction of apoptosis. First, the frequency of apoptotic and necrotic cells in the presence of bortezomib was determined using Annexin V-FITC and propidium iodide staining. The percentages of Annexin V-FITC-positive/propidium iodide-negative apoptotic cells were significantly higher in Ag8μH+ compared with Ag8μH− subclones (Fig. 2B). Second, induction of apoptotic cell death by bortezomib was verified by measuring the mitochondrial membrane potential ψ using the fluorescent dye DiOC6. The mean fluorescence intensity was markedly lower in Ag8μH+ cells, again confirming that bortezomib preferentially induced apoptosis in Ag8 cells expressing μH-chains (Fig. 2C). Third, there was significantly higher caspase-3/7 activity in Ag8μH+ compared with Ag8μH− subclones (Fig. 2D). To evaluate the functional importance of caspase activation for bortezomib-induced apoptosis, we analyzed the influence of the pan-caspase inhibitor zVAD-FMK. As shown in Fig. 2E, zVAD-FMK significantly interferes with bortezomib-triggered cell death in Ag8μH+ subclones. These results based on the analyses of various subclones with and without μH-chain expression, all derived from the same parental μH-chain–transfected Ag8 clone, and therefore, only differing in immunoglobulin expression, further confirmed that immunoglobulin synthesis is sufficient to confer increased sensitivity towards apoptosis upon proteasome inhibition.

**Bortezomib induces proapoptotic CHOP in Ag8μH+ cells.** Next, we wanted to further investigate the mechanism of apoptosis induction by bortezomib in Ag8μH+ cells. Recently, it has been shown that inhibition of the proteasome induces UPR. Hence, we tested whether bortezomib triggers ER stress in Ag8 subclones, leading to activation of terminal UPR and apoptosis. We analyzed prominent UPR components, such as BiP, XBP-1, CHOP, and caspase-12, known to be activated by classic inducers of UPR such as the glycosylation inhibitor tunicamycin.

First, we evaluated the activation of the prosurvival chaperone BiP over time in bortezomib-treated Ag8μH− compared with Ag8μH+ cells. Immunoblotting showed that the basal levels of BiP were already very high, and neither tunicamycin nor bortezomib further increased protein levels of BiP in Ag8μH− and Ag8μH+ cells (Fig. 3A). Next, we studied the influence of bortezomib on the expression of XBP-1, a transcription factor inducing UPR components including chaperones. Upon ER stress, XBP-1 mRNA is spliced by the stress transmembrane kinase/Nase IRE1 to generate a transcript encoding a XBP-1 protein with higher transactivator activity. This splicing event is a frameshift splicing resulting in the translation of a larger XBP-1 protein (28). Bortezomib treatment diminished the amount of spliced XBP-1 mRNA both in Ag8μH− and Ag8μH+ cells (Fig. 3B), whereas the spliced and unspliced form of the XBP-1 protein markedly increased upon bortezomib treatment in all subclones (Fig. 3C). This discrepancy is most likely explained by decreased proteolysis of XBP-1 during proteasome blockade, as described previously (29).

Next, we investigated whether bortezomib induces UPR proteins involved in mediating apoptosis. Overexpression of CHOP (30), along with activation of caspase-12 (31), is proposed to foster apoptotic processes within the UPR. Bortezomib treatment of Ag8 cells resulted in a marked induction of CHOP at 8 h (Fig. 3A). Interestingly, in Ag8μH+ cells, CHOP accumulated earlier (4 h)
Figure 2. Bortezomib induces cell death in Ag8H+-/+ subclones. A, cell cycle analysis. Ag8H+-/+ cells were treated with 1 μM/L bortezomib for indicated time intervals. DNA content of cells incubated with propidium iodide/Triton X-100 was determined by flow cytometry. One representative histogram out of triplicates. B, cell death analysis. Ag8H+-/+ subclones treated with 1 μM/L bortezomib (Bz) for indicated time intervals were stained with Annexin V-FITC/propidium iodide (PI) and analyzed by flow cytometric analysis. Diagrams show the percentages of Annexin V-FITC–positive (+)/propidium iodide–negative (−) and Annexin V-FITC/propidium iodide double-positive cells of Ag8H− versus Ag8H+ subclones. C, measurement of mitochondrial membrane potential. Ag8H+-/+ subclones were treated with 1 μM/L bortezomib for indicated time intervals. Cells were double stained with DiOC6 dye and propidium iodide and analyzed by flow cytometry. Mean fluorescence intensities (MFI) of propidium iodide–negative/DiOC6–positive cells are displayed in the diagram, with high MFI indicating non-apoptotic cells. D, analysis of caspase-3/7 activation. Ag8H+-/+ subclones were treated with 1 μM/L bortezomib for 16 h. Activation of caspase-3/7 was determined using the Caspase-Glo assay. Columns, mean of luminescence in RLU; bars, SD. E, caspase inhibition. Ag8H+-/+ subclones were treated with 1 μM/L bortezomib in the presence or absence of the caspase inhibitor ZVAD-FMK for 2 h, or inhibitor alone. Cells were stained with Annexin V-FITC/propidium iodide and analyzed by flow cytometry. Diagrams show the percentages of Annexin V-FITC–positive/propidium iodide–negative and Annexin V-FITC/propidium iodide double-positive cells of Ag8H− versus Ag8H+ subclones. Columns, mean from triplicates in all experiments; bars, SD. **, P < 0.01; *, P < 0.05.

quantitative real-time PCR analyses revealed a reduction of Bcl-2 mRNA in Ag8H+–/– cells upon bortezomib treatment; in Ag8H+ subclones, there was no marked change of Bcl-2 mRNA levels observed (Fig. 4A). However, Bcl-2 protein levels decreased to a similar extent in both cell lines at 4 and 8 h after treatment with bortezomib (Fig. 4B).

In Ag8H− cells, the mRNA levels of the proapoptotic factor Bax slightly decreased upon bortezomib treatment. In contrast, in Ag8H+ cells, Bax mRNA levels increased, especially 2 h after bortezomib treatment (Fig. 4A). In agreement with real-time PCR results, Bax protein concentrations decreased in Ag8H− cells but accumulated over a period of 8 h after addition of bortezomib in Ag8H+ cells (Fig. 4B). In summary, the ratio of Bcl-2/Bax proteins as calculated by densitometric analyses (data not shown) was lower in Ag8H+ cells compared with Ag8H− cells 8 h after bortezomib treatment. This differential regulation by bortezomib of antiapoptotic versus proapoptotic Bcl-2 family members may contribute to increased apoptotic cell death in Ag8H+ compared with Ag8H− cells.

Bortezomib alters activity of the transcription factors NF-κB and activator protein-1. Because it is generally assumed that the antitumor effect of bortezomib is, at least partially, due to inhibition of NF-κB (34), we analyzed the basal NF-κB activity in Ag8 myeloma cells. Nuclear extracts prepared from bortezomib-treated Ag8H+ and Ag8H− cells were subjected to EMSA to quantify NF-κB DNA-binding activity. In
Acetylation, and phosphorylation events. Therefore, we analyzed regulated by other mechanisms including complex composition, does not necessarily reflect transcriptional activity, which is also treated with 5 μM tunicamycin (tun) for 6 h. Cell lysates were separated by SDS-PAGE on a 10% polyacrylamide gel, and immunoblotting was done using anti-BIP, anti-XBP-1 (u, unspliced; s, spliced), and anti-CHOP antibodies. Anti-actin antibody staining served as control for equal loading. B, reverse transcription-PCR analysis of total RNA isolated from Ag8H+ versus Ag8H− subclones treated with 1 μM tunicamycin for indicated time intervals. Tunicamycin-treated cells served as positive control. Reverse transcription-PCR was done using a primer set that detects the unspliced (XBP-1u, 442 bp) and spliced (XBP-1s, 416 bp) form of XBP-1 mRNA. PCR products were resolved on a 2% agarose gel. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used for adjustment of the mRNA concentrations. One of two independently done experiments. C, caspase-12 activation by bortezomib in Ag8H−/− subclones. Western blot analysis of total cell lysates from Ag8H−/− subclones treated with 1 μM tunicamycin for indicated time intervals was done using an anti-caspase-12 antibody. Pro-caspase-12 cleavage products indicating activation of caspase-12 were detectable only in Ag8H−/− cells, most pronounced after 12 h of bortezomib treatment. One representative immunoblot of three independent experiments.

Repeated experiments we did not find major differences in DNA binding (Fig. 5A and B). If at all, upon bortezomib treatment, there was a trend towards a decreased NF-κB DNA-binding activity in Ag8H− subclones but an increased NF-κB activity in Ag8H+ (Fig. 5A and B). However, NF-κB DNA-binding activity does not necessarily reflect transcriptional activity, which is also regulated by other mechanisms including complex composition, acetylation, and phosphorylation events. Therefore, we analyzed IκBα mRNA levels, which are strongly induced by NF-κB activation (35). Relative IκBα mRNA levels dropped in both Ag8 subclones upon bortezomib treatment (Fig. 5C), indicating that NF-κB transcriptional activity is reduced at a similar degree in Ag8H+ and Ag8H− subclones. Hence, preferential blockade of the survival factor NF-κB does not account for the increased sensitivity of Ag8H+ cells towards bortezomib. It remains unclear which mechanisms cause the discrepancy between DNA-binding activity and transcriptional activity of NF-κB under conditions of proteasome blockade.

Interestingly, DNA-binding activity of the transcription factor Oct-1 slightly increased in Ag8H+ and Ag8H− subclones upon bortezomib treatment (Fig. 5A and B), which might be due to stabilization of the transcription factor because of reduced proteasomal degradation. However, DNA-binding activity of the transcription factor activator protein-1 (AP-1), which is involved in repeated experiments we did not find major differences in DNA binding (Fig. 5A and B). If at all, upon bortezomib treatment, there was a trend towards a decreased NF-κB DNA-binding activity in Ag8H− subclones but an increased NF-κB activity in Ag8H+ (Fig. 5A and B). However, NF-κB DNA-binding activity does not necessarily reflect transcriptional activity, which is also regulated by other mechanisms including complex composition, acetylation, and phosphorylation events. Therefore, we analyzed IκBα mRNA levels, which are strongly induced by NF-κB activation (35). Relative IκBα mRNA levels dropped in both Ag8 subclones upon bortezomib treatment (Fig. 5C), indicating that NF-κB transcriptional activity is reduced at a similar degree in Ag8H+ and Ag8H− subclones. Hence, preferential blockade of the survival factor NF-κB does not account for the increased sensitivity of Ag8H+ cells towards bortezomib. It remains unclear which mechanisms cause the discrepancy between DNA-binding activity and transcriptional activity of NF-κB under conditions of proteasome blockade.

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Figure 3. Bortezomib induces UPR components. A, Western blot analysis of total cell lysates from Ag8H−/− subclones treated with 1 μM/L bortezomib for indicated time intervals. As a positive control for UPR activation, cells were treated with 5 μg/mL tunicamycin (tun) for 6 h. Cell lysates were separated by SDS-PAGE on a 10% polyacrylamide gel, and immunoblotting was done using anti-BIP, anti-XBP-1 (u, unspliced; s, spliced), and anti-CHOP antibodies. Anti-actin antibody staining served as control for equal loading. B, reverse transcription-PCR analysis of total RNA isolated from Ag8H+ versus Ag8H− subclones treated with 1 μM/L bortezomib for indicated time intervals. Tunicamycin-treated cells served as positive control. Reverse transcription-PCR was done using a primer set that detects the unspliced (XBP-1u, 442 bp) and spliced (XBP-1s, 416 bp) form of XBP-1 mRNA. PCR products were resolved on a 2% agarose gel. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used for adjustment of the mRNA concentrations. One of two independently done experiments. C, caspase-12 activation by bortezomib in Ag8H−/− subclones. Western blot analysis of total cell lysates from Ag8H−/− subclones treated with 1 μM/L bortezomib for indicated time intervals was done using an anti-caspase-12 antibody. Pro-caspase-12 cleavage products indicating activation of caspase-12 were detectable only in Ag8H−/− cells, most pronounced after 12 h of bortezomib treatment. One representative immunoblot of three independent experiments.

Figure 4. Bortezomib modulates expression of Bcl-2 family members. A, quantitative real-time PCR analysis of total RNA isolated from Ag8H−/− subclones treated with 1 μM/L bortezomib for indicated time intervals. As a control, cells were treated with 5 μg/mL tunicamycin (tun) for 6 h. Relative Bcl-2 and Bax mRNA levels of bortezomib-treated Ag8H−/− subclones normalized to β-actin mRNA levels, which served as internal control. cDNA from untreated cells served as reference control. The comparative Ct (ΔΔCt) method for relative quantification of gene expression was used. PCR analysis was done in triplicates. One representative data set of at least two independent experiments. B, Western blot analysis of total cell lysates from Ag8H−/− subclones, cultured for the indicated time intervals in the presence of 1 μM/L bortezomib. Tunicamycin-treated cells served as control. Cell lysates were separated by SDS-PAGE on a 12.5% polyacrylamide gel. Immunoblotting was done using anti-Bcl-2 and anti-Bax antibodies. The upper bands detected by the Bcl-2 antibody are proposed to be phosphorylated forms of Bcl-2, and the lower one, running at 23 kDa, corresponds to a cleaved form of Bcl-2. Anti-actin antibody staining served as loading control.
In the presence of bortezomib, we observed increased intracellular activity, proliferation, and stress-induced apoptosis (36), was markedly induced selectively in Ag8 myeloma cells (Fig. 5A and B). These data may indicate a role of ER overload/stress for induction of AP-1.

IgG-derived DRiPs accumulate in response to proteasome inhibition. It has been shown that due to failure at translation or posttranslational processes, ~30% of newly synthesized proteins are defective (26, 37). In myeloma cells, the relatively high expression levels of immunoglobulin should concomitantly result in the formation of a substantial number of ER-resident DRiPs, which ultimately have to be degraded by the ubiquitin-proteasome system. Therefore, proteasome inhibition should cause an accumulation of DRiPs and other unfolded proteins responsible for induction of UPR-initiated apoptosis.

To analyze IgG DRiP formation in a human non-transfected myeloma cell line, we did short-term pulse-chase experiments using JK-6L cells. To rescue defective ribosomal products from proteasomal degradation, we used a combination of two proteasome inhibitors (lactacystin and MG-132), which in previous DRiP pulse-chase experiments have been shown to efficiently and rapidly shut down the activity of the 26S proteasome. 35S-methionine-labeled IgG molecules were recovered from the soluble fraction by immunoprecipitation using anti-human IgG antibodies. Upon proteasome inhibition, the recovery of radiolabeled IgG heavy chains was increased by ~30%, reaching its maximum at 5 min after pulse radiolabeling (Fig. 6A). These results are consistent with an accumulation of IgG-DRiPs caused by proteasome inhibition.

Discussion

Bortezomib was previously shown to target malignant myeloma cells through induction of apoptosis, probably by blocking NF-κB activation, which mediates survival and resistance to chemotherapy and radiotherapy (2, 6, 7, 38, 39). However, testing numerous cell lines for their basal NF-κB activity and their sensitivity to proteasome inhibition, we previously observed that high levels of protein synthesis were more predictive for proteasome inhibitor–induced apoptosis than NF-κB activity (data not shown). In addition, previous reports evidenced that proteasome inhibition has more profound effects on myeloma cells than just NF-κB inhibition (29, 40, 41), indicating mechanisms beyond NF-κB blockade.

Extensive protein synthesis within the secretory ER pathway is an extraordinary feature of plasma cells and their malignant counterparts, myeloma cells. In the case of secreted proteins, such as immunoglobulin, DRiPs should initially arise in the ER, whereas DRiP degradation is proposed to take place in the cytoplasmic proteasomes. Therefore, we suggest that accumulation of unfolded proteins, predominantly DRiPs, within the ER leads to UPR activation, resulting in inhibition of protein synthesis and induction of ER chaperones to enhance folding and to prevent aggregation of nascent secretory proteins. Unfolded proteins that cannot be correctly folded are redirected to the cytoplasm by the action of ERAD and degraded by the 26S proteasome (42). Blockade of proteasomes inhibits consequently ERAD-mediated export of unfolded proteins into the cytoplasm, thereby increasing UPR and ER overload (42). Whereas UPR activation is essential in plasma cell physiology and survival (14–16), prolonged overwhelming UPR activation causes apoptosis. Hence, myeloma cells producing enormous numbers of immunoglobulin should accumulate deleterious amounts of unfolded proteins under condition of proteasome inhibition.

In the presence of bortezomib, we observed increased intracellular amounts of μH-chains, which were predominantly ubiquitylated, indicating a proteasome-dependent degradation of unfolded μH-chains (data not shown). Other groups also provided


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evidence that non-secreted ER-retained IgL- and μH-chains are degraded by the ubiquitin-proteasome pathway (43, 44). Thus far, all DRiPs assigned to a defined protein were either derived from infected or transfected cells overexpressing viral or recombinant proteins but not from endogenously expressed proteins (26, 45, 46). Pulse-chase analyses revealed an accumulation of 35S-methionine–labeled IgG in the cytoplasmic fractions. Accumulation of DRiPs due to proteasome inhibition may be an important mechanism of bortezomib-mediated cell death, targeting primarily cells with high protein synthesis. Of course, this represents not the only cytotoxic mechanism of bortezomib. For instance, other tumor cells may be primarily eliminated due to their dependence on NF-κB activation.

A very recent report comparing two cell lines from different patients found an increased sensitivity towards bortezomib, the non–immunoglobulin-producing murine myeloma cell line Ag8.H was stably transfected with a μH-chain expression plasmid. Expression of μH-chain resulted in drastically increased sensitivity to proteasome inhibition. Most likely, increased synthesis of ER proteins generally leads to increased susceptibility against proteasome inhibitors, as evidenced by transgenic expression of the transmembrane protein Igα.

To exclude transfection artifacts, subclones of the Ag8.H clone were established. In addition, Ag8 μH+ subclones were much more sensitive to proteasome inhibitors compared with Ag8 μH– subclones. Cell death induced by bortezomib was predominantly apoptotic, both in Ag8 μH+ and Ag8 μH– cells as shown by Annexin V-FITC/propidium iodide staining, decreased mitochondrial membrane potential, and caspase activation. As expected, the pan-caspase inhibitor zVAD-FMK significantly delayed onset of cell death.

Figure 6. Proteasome inhibitors induce accumulation of IgG DRiPs. A, JK-6L cells treated with 25 μmol/L MG-132/lactacystin (LC) or DMSO as a solvent control were radiolabeled for 5 min with 35S-methionine and chased for up to 30 min. IgG molecules were recovered from CHAPS-deoxycholate–solubilized cell lysates by immunoprecipitation and analyzed by SDS-PAGE. 35S-radioactivity of IgG heavy chains was quantified using phosphoimaging and plotted as percentage of initial incorporation.

B, model of bortezomib-induced apoptosis in myeloma cells. In cells secreting large amounts of immunoglobulin, defective ribosomal products and other unfolded proteins arise, which get ubiquitinated and are normally degraded by the 26S proteasome complex. Proteasome inhibitors, such as bortezomib, block the enzymatic activity of proteasomes, thereby preventing disposal of misfolded proteins and NF-κB activation. Consequently, accumulation of unfolded proteins/DRiPs within the cell along with weakened activation of the survival factor NF-κB, which is otherwise strongly induced by ER overload, leads to apoptosis.
Reactive oxygen species (ROS) are involved in the process of bortezomib-induced apoptotic cell death (47). As described, ROS, produced by misfolded proteins (oxidation and reduction of disulfide bonds), accumulate during prolonged UPR and contribute to cell death (48). We observed a significantly higher increase of ROS by bortezomib treatment in Ag8–H+ compared with Ag8–H− subclones, linking increased ROS production and susceptibility to cell death to immunoglobulin synthesis (data not shown). In addition, elimination of ROS by antioxidants, such as N-acetylcysteine, markedly reduced bortezomib-induced cell death in Ag8–H+ cells (data not shown).

In spite of the high number of subclones analyzed, we could not identify any IgG-negative JK-6L subclones, indicating that immunoglobulin expression is crucial for survival of plasma as well as myeloma cells, most likely by UPR-mediated expression of chaperones and autocrine growth factors such as IL-6 (14–16). Extensive studies have elucidated the relationship between the UPR and plasma cell differentiation (49). The UPR-induced transcription factors XBP-1 and ATF6 mediate the induction of UPR target genes such as ER-resident chaperones.

One major mechanism by which bortezomib induces apoptosis in Ag8 cells could be the induction of the terminal UPR pathway, driven by overwhelming accumulation of defective immunoglobulin chains in the ER. Previously published data have shown that bortezomib is able to induce ER stress in pancreatic cancer and myeloma cells (29, 40, 41). To investigate whether proteasome inhibition activates UPR, and whether this depends on immunoglobulin synthesis, we analyzed the induction and activation status of prosurvival UPR components, such as BiP and proapoptotic terminal UPR components, such as CHOP and caspase-12, in Ag8–H− versus Ag8–H+ subclones. Chaperones like BiP are highly constitutively expressed in plasma cells to ensure the secretory cell function. Our finding that BiP synthesis is not markedly altered in bortezomib- and tunicamycin-treated Ag8 cells suggests that BiP expression has already reached maximally high levels in these myeloma cells.

UPR-mediated cell survival or cell death is regulated by the balance of cytoprotective and proapoptotic factors (17). Consistent with findings of other groups (40, 41, 50), we showed that CHOP is strongly up-regulated in bortezomib-treated cells. Most importantly, our data revealed a faster and stronger induction of CHOP in Ag8–H+ compared with Ag8–H− cells. Hence, bortezomib causes a strong up-regulation of CHOP without an increase of BiP, thereby fostering apoptotic cell death by altering the ratio of these prosurvival and antisurvival factors.

One relevant target of CHOP is Bcl-2, whose expression is suppressed by CHOP (32). Bcl-2 protein levels were clearly reduced during incubation with bortezomib, but in Ag8–H+ cells, Bcl-2 protein expression partially recovered after 8 h, a fact that may reflect a block in proteasome-mediated degradation of Bcl-2 (51, 52). Based on our findings, the previously observed bortezomib-induced reduction of Bcl-2 (53) may be, at least partially, mediated by increased CHOP levels.

The proapoptotic Bax, antagonizing Bcl-2, was induced on mRNA and protein level in bortezomib-treated Ag8–H+ but not in Ag8–H− cells. These data suggest transcriptional induction of Bax; however, we cannot exclude additional effects of bortezomib on proteasome-mediated degradation of Bax (54). In Ag8–H+ cells, bortezomib modulates the Bcl-2 to Bax ratio in favor of the proapoptotic Bax, whereas in Ag8–H− cells, the Bcl-2 to Bax ratio is altered more in favor of the antiapoptotic Bcl-2. In summary, bortezomib-induced proapoptotic factors, such as Bax, CHOP, and caspases, may dominate over antiapoptotic factors like Bcl-2 in tilting the balance towards apoptosis, especially in immunoglobulin-synthesizing cells.

Inhibition of NF-κB activation by bortezomib is generally considered as a major mechanism of the proapoptotic effects of proteasome inhibition on tumor cells (7). In addition, UPR and ER overload have been shown to induce activation of NF-κB (55, 56). As expected, we did not observe a marked increase of NF-κB DNA-binding activity under conditions of bortezomib-induced ER stress, most likely due to simultaneous blockade of proteasome-dependent IκB degradation. Monitoring IκB-Bo mRNA expression, which is highly dependent on NF-κB activity, even indicated a marked reduction in NF-κB transcriptional activity in both Ag8 subclones. The discrepancy in between just slightly changed NF-κB DNA-binding activity and drastically reduced transcriptional activity remains still elusive but might be due to altered posttranslational modifications and coactivator recruitment to NF-κB. We conclude that inhibition of NF-κB activation is not the crucial factor, which explains the differential sensitivity of Ag8–H+ versus Ag8–H− subclones to proteasome inhibition: NF-κB DNA-binding and transcriptional activity were similarly affected in Ag8–H+ and Ag8–H− cells, if at all, there was a even stronger inhibition in Ag8–H− clones. Nevertheless, inhibition of NF-κB activity may contribute to apoptosis.

The transcription factor AP-1 was significantly induced in bortezomib-treated Ag8–H+ cells. AP-1 is induced by the UPR kinase IRE-1 via activation of c-Jun NH2-terminal kinase (JNK), which is known to mediate stress-induced apoptosis (36, 57). Therefore, JNK/AP-1 signaling may also contribute to bortezomib-induced cell death.

This work shows that myeloma cells producing high amounts of immunoglobulin are more susceptible to apoptosis by agents, such as bortezomib, that evoke overwhelming ER stress by inhibiting the degradation of unfolded proteins, including DRiPs. Simultaneously, bortezomib interferes with ER overload–mediated activation of NF-κB, which normally counteracts ER stress–induced apoptosis (Fig. 6B).

Our findings that sensitivity towards proteasome inhibition is primarily dependent on the extent of immunoglobulin synthesis have important implications for the therapeutic use of bortezomib and other proteasome inhibitors. We postulate that tumors synthesizing high amounts of proteins, especially secreted ones, represent primary targets of proteasome inhibitors. Down-regulation of protein biosynthesis or increase of protein folding enzymes/chaperones could represent a novel mechanism for partial resistance towards bortezomib. However, a complete cessation of immunoglobulin production may be barely observed because a significant level of UPR activation seems to be important for plasma and myeloma cell physiology and survival (14–16). Moreover, agents influencing protein folding and DRiP rate may modulate the sensitivity of cells towards proteasome inhibitors and augment the antimonyeloma activity of bortezomib.

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Extensive Immunoglobulin Production Sensitizes Myeloma Cells for Proteasome Inhibition


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