Mitochondrial-Mediated Disregulation of Ca\textsuperscript{2+} Is a Critical Determinant of Velcade (PS-341/Bortezomib) Cytotoxicity in Myeloma Cell Lines

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Abstract
The proteasome inhibitor bortezomib (also known as PS-341/Velcade) is a dipeptidyl boronic acid that has recently been approved for use in patients with multiple myeloma. Bortezomib inhibits the activity of the 26S proteasome and induces cell death in a variety of tumor cells; however, the mechanism of cytotoxicity is not well understood. In this report, oligonucleotide microarray analysis of the 8226 multiple myeloma cell line showed a predominant induction of gene products associated with the endoplasmic reticulum secretory pathway following short-term, high-dose exposure to bortezomib. Examination of mediators of endoplasmic reticulum stress–induced cell death showed specific activation of caspase 12, as well as of caspases 8, 9, 7, and 3, and cleavage of bid. Treatment of myeloma cells with bortezomib also showed disregulation of intracellular Ca\textsuperscript{2+} as a mechanism of caspase activation. Cotreatment with a panel of Ca\textsuperscript{2+}-modulating agents identified the mitochondrial uniporter as a critical regulatory factor in bortezomib cytotoxicity. The uniporter inhibitors ruthenium red and Ru360 prevented caspase activation and bid cleavage, and almost entirely inhibited bortezomib-induced cell death, but had no effect on any other chemotherapeutic drug examined. Additional Ca\textsuperscript{2+}-modulating agents, including 2-amino-ethoxydiphenylborate, 1,2-bis (o-aminophenoxy) ethane-tetraacetic acid (acetoxy-methyl) ester, and dantrolene, did not alter bortezomib cytotoxicity. Analysis of intracellular Ca\textsuperscript{2+} showed that the ruthenium-containing compounds inhibited Ca\textsuperscript{2+} store loading and abrogated the desensitized capacitative calcium influx associated with bortezomib treatment. These data support the hypothesis that intracellular Ca\textsuperscript{2+} disregulation is a critical determinant of bortezomib cytotoxicity. (Cancer Res 2005; 65(9): 3828-36)

Introduction
Bortezomib (PS-341/Velcade) is a dipeptide containing boronic acid, which has been recently approved for therapeutic use in refractory multiple myeloma (1, 2). Bortezomib forms a covalent bond with the active site threonine in the core of the 20S proteasome and inhibits the chymotryptic activity of the proteasome; however, its exact mechanism of cytotoxicity and selectivity for transformed cells is not known. A large number of proteins that are involved with carcinogenesis are known to be regulated by the ubiquitin-proteasome system of degradation, including transcription factors such as activator protein-1 and p53, signal transduction molecules such as Jak2 and cbl, the cell cycle regulators p21 and p27, and regulatory factors such as the nuclear factor-κB inhibitors IκBα, β, ε, and p100. Many laboratories have investigated these proteins as potential targets of bortezomib-mediated cytotoxicity, and these studies have clearly shown that bortezomib is a potent inhibitor of the 26S proteasome with effects that can be directly related to protein stabilization (3–5). However, the molecular switch that initiates cell death in this pathway has not yet been identified.

Over the past 10 years, two primary apoptotic pathways have been described. The “extrinsic pathway” is initiated by ligation of cell-surface death receptors such as CD95 (Fas/Apo-1), tumor necrosis factor receptor 1, and death receptors 4 and 5 (see review in ref. 6). Upon receptor ligation, the adapter protein Fas-associated death domain protein is recruited, which in turn recruits procaspase 8, resulting in the formation of the death-inducing signaling complex (8). Death-inducing signaling complex formation is generally thought to initiate apoptosis by induced proximity autocatalytic activation of caspase 8 and subsequent downstream effectors. In contrast, the “intrinsic pathway” is associated with various cell damaging agents such as reactive oxygen species and DNA strand breaks, and is initiated by mitochondrial release of cytochrome c, formation of the apoptosome, and activation of procaspase 9. Cross talk between these two pathways occurs through the Bcl-2 family, which includes both proapoptotic and antiapoptotic members (7). For example, bid is a proapoptotic factor that has been shown to be cleaved by caspase 8, releasing an activated fragment, which can induce mitochondrial release of cytochrome c. Bcl-x\textsubscript{L} is an antiapoptotic factor that has been shown to inhibit cell death induced by both death receptor ligation and cytotoxic drugs in some cell types (8). Thus, bid activation by caspase 8 and mitochondrial release of cytochrome c are thought to amplify extrinsic apoptotic signals by recruiting involvement of the mitochondrial pathway.

More recently, a third pathway has been identified that is initiated by the endoplasmic reticulum (ER; see review in ref. 9). At least two different mechanisms have been associated with ER stress–initiated apoptosis, the unfolded protein response and disregulation of Ca\textsuperscript{2+} homeostasis.

Disregulation of intracellular Ca\textsuperscript{2+} was among the first hallmark of apoptosis, predating the identification of the caspase cascade as a mechanism of programmed cell death (see review in ref. 10). Early work identified a biphasic increase in cytosolic Ca\textsuperscript{2+} associated with apoptosis: first, a transient spike occurring immediately following the cellular insult; second, sustained Ca\textsuperscript{2+} influx that was considered to be the lethal event. Subsequent studies have shown that even minor disruptions in either total Ca\textsuperscript{2+} or subcellular distribution can modulate the apoptotic

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Ca²⁺ Disregulation by Bortezomib

response to a large number of stimuli. In most cell types, the ER is the primary intracellular store of Ca²⁺, where it participates in the folding, modification, and sorting of newly synthesized proteins. Homeostasis between Ca²⁺ stores and cytosolic Ca²⁺ is maintained by ER-resident channels and transporters. The primary mechanism for ER Ca²⁺ influx is the smooth ER Ca²⁺-ATPase, which mediates store filling. Two primary Ca²⁺-sensitive channels mediate the release of Ca²⁺ from the ER, the inositol triphosphate receptor (IP3R) and the caffeine sensitive ryanodine receptor. Recent studies suggest that IP3R-activated depletion of ER stores is also involved in the regulation of Ca²⁺ influx from the extracellular environment, a phenomenon known as capacitative Ca²⁺ influx (CCI; ref. 11). Vazquez et al. showed that a genetic variant of DT40 B lymphocytes deficient in IP3R expression was unable to transport Ca²⁺ into the cell and was resistant to apoptosis following B-cell receptor ligation. Transfection of IP3R expression constructs restored this capability; however, the identity of the plasma membrane channel activated by elevated inositol triphosphate and the mechanism of IP3R regulation remain under investigation. Additional studies have also implicated IP3R-mediated CCI as a modulator of apoptosis (12–14). Jayaraman et al. showed that transfection of T lymphocytes with IP3R antisense rendered the cells resistant to apoptotic cell death induced by dexamethasone, ionizing radiation, T-cell receptor ligation, and CD95 cross-linking. This resistance could be overcome by pharmacologically increasing cytosolic Ca²⁺, supporting a role for CCI in apoptosis.

In addition to their function in metabolism and apoptosis, mitochondria are known to participate in Ca²⁺ homeostasis. Excess cytosolic Ca²⁺ is taken up into the mitochondria through a low-affinity inner membrane Ca²⁺ uniporter and released back to the cytosol by three independent mechanisms: reversal of the uniporter, Na⁺/H⁺-dependent Ca²⁺ exchange, or the mitochondrial permeability transition pore opening. Additional studies have shown IP3R-mediated translocation of Ca²⁺ to mitochondria following treatment with staurosporine or ceramide, resulting in rapid permeability transition pore opening, cytochrome c release, and activation of downstream mediators of apoptosis (15). In these studies, apoptosis could be inhibited by ruthenium red, a cationic dye that blocks the mitochondrial uniporter, or by bongkrekic acid, which binds to the adenine nucleotide translocator and prevents permeability transition pore opening. More recently, several studies have shown a role for the Bcl-2 family of apoptotic regulators in the translocation of Ca²⁺ from the ER to the mitochondria (16, 17). Collectively, these studies show an additional mechanism of cross talk between the known apoptotic pathways and emphasize the complex coordination of signals that contribute to cell survival or death.

In the present study, we have examined the early events associated with bortezomib-induced cell death of multiple myeloma cells. Multiple myeloma is a malignancy of secretory plasma cells, and as such, they contain a highly developed ER, characteristic of secretory cells. We show that bortezomib treatment induces the expression of proteins associated with the ER secretory pathway, activation of caspase 12, and deregulation of Ca²⁺ homeostasis leading to cell death. We further show that inhibition of mitochondrial Ca²⁺ uptake by ruthenium compounds completely abrogates the cytotoxic activity of bortezomib, whereas a series of Ca²⁺-modulating agents has no significant effect. These results suggest that bortezomib initiates apoptosis in myeloma cells by a unique mechanism that is not known to be activated by any other chemotherapeutic agent.

Materials and Methods

Cells and antibodies. The 8226, H929, and U266 myeloma cell lines were originally obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA), supplemented with 5% or 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 1 mmol/L l-glutamine, and 100 units/ml penicillin/streptomycin (Gibco/Invitrogen). The MM.1S cell line was kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL; ref. 18). Caspase 3, 8, 9, and 12 antibodies were obtained from Cell Signaling (Beverly, MA).

Oligonucleotide expression analysis. 8226 myeloma cells were exposed to 50 mmol/L bortezomib for 4 hours and harvested for isolation of RNA using the Qiagen Rneasy protocol followed by Oligotex mRNA kit (Qiagen, Valencia, CA). Three independent experiments were done on consecutive days, and equal quantities of RNA from each experiment were combined for oligonucleotide microarray analysis using the Affymetrix U113A gene chip, which includes oligonucleotides representative of 22,000 gene products. Data were analyzed using GeneChip software as previously described (19). Untreated cells were designated as the baseline and comparison metrics calculated to identify differences between the baseline and the cells treated with bortezomib.

Cytotoxicity assays. Cytotoxicity analysis was done either by Annexin V-FITC/Mito Tracker red (CMXRos, Molecular Probes, Eugene, OR) staining and flow cytometry or by using the 3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as previously described (20). For all studies using combinations of a Ca²⁺ inhibitor plus cytotoxic agent, the inhibitor was added to the total cell population before plating.

Cytotoxic activity of the Ca²⁺-modulating agents alone was determined in preliminary control experiments, and all subsequent assays were done at minimally toxic doses as follows: 2-amino-ethoxydiphenylborate (2-APB), 5 μmol/L; 1,2-bis (o-aminophenoxy) ethane-tetracetic acid (acetoxyethyl) ester (BAPTA-AM), 10 μmol/L; dantrolene, 10 μmol/L; Ru360, 1.0 μmol/L; and ruthenium red, 0.1 μmol/L. All reagents were obtained from Calbiochem (San Diego, CA) with the exception of ruthenium red, which was obtained from Sigma (St. Louis, MO). Bortezomib (PS-341/Velcade) was kindly provided by Millennium Pharmaceuticals (Cambridge, MA).

Immunoblot analysis. Immunoblotting was done as previously described (19). Briefly, after drug treatment, cells were pelleted by centrifugation, washed once with ice-cold PBS, and lysed in 30 mmol/L HEPES (pH 7.5), 10 mmol/L NaCl, 5 mmol/L MgCl₂, 25 mmol/L NaF, 1 mmol/L EGTA, 1% Triton X-100, 10% glycerol with 2 mmol/L NaVO₄, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, and 2 mmol/L phenylmethylsulfonil fluoride for 20 minutes on ice. Lysates were cleared by centrifugation and protein was quantitated by bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein (30-50 μg) were separated by SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), probed with the specified antibody, and developed using Pierce Supersignal chemiluminescence substrate.

[Ca²⁺]ᵢ measurements. For [Ca²⁺]ᵢ measurements, cells were seeded at 1 × 10⁴ cells/ml in 24-well plates in complete medium supplemented with 5% or 10% heat-inactivated fetal bovine serum and incubated for 24 hours. Individual wells were incubated in a buffered saline (5 mmol/L KCl, 0.3 mmol/L KH₂PO₄, 138 mmol/L NaCl, 0.2 mmol/L NaHCO₃, 0.3 mmol/L Na₂HPO₄, 20 mmol/L HEPES, 1.3 mmol/L CaCl₂, 0.4 mmol/L MgSO₄, and 5.6 mmol/L glucose, pH 7.3, at 37°C) containing 2.5 μmol/L Fura-PE3/AM (Teflabs, Austin, TX) with identical results on both substrates. Individual coverslips were incubated in a buffered saline (5 mmol/L KCl, 0.3 mmol/L KH₂PO₄, 138 mmol/L NaCl, 0.2 mmol/L NaHCO₃, 0.3 mmol/L Na₂HPO₄, 20 mmol/L HEPES, 1.3 mmol/L CaCl₂, 0.4 mmol/L MgSO₄, and 5.6 mmol/L glucose, pH 7.3, at 37°C) containing 2.5 μmol/L Fura-PE3/AM (Teflabs, Austin, TX) with 0.0025% pluronic acid for 20 minutes at 37°C. The coverslips were then rinsed in HBSS for 20 minutes at 37°C to allow full hydrolysis of the Fura-AM to free acid. The coverslip with the dye-loaded cells was then placed in a 37°C imaging chamber, which is mounted on the stage of an inverted Olympus IX70 microscope (Melville, NY). Light emitted from a 100-W Hg bulb was passed alternately through 340- and 380-nm filters using a filter wheel. The emitted fluorescence was collected using a 40× 1.35 numerical aperture UV-fluor objective then selected using a 10-nm bandpass filter centered at 510 nm and passed to a CCD camera.
The ratio of the light intensity monitored at 340/380 is used as an index for [Ca^{2+}]_i. Specific calibration procedures have been previously reported in detail (21). A minimum of 5 cells was analyzed in each experimental condition, and all experiments were repeated at least thrice. In the standard protocol, Ca^{2+} was removed from the media for 2 minutes before addition of cyclopiazic acid, then peak changes in Ca^{2+} following cyclopiazic acid and Ca^{2+} addition were calculated as percent change from the zero Ca^{2+} baseline (baseline) using the formula \[(R_{\text{peak}} \text{ following treatment}) - (R_{\text{immediately before treatment}}) / (R_{\text{peak immediately before treatment}}, \text{baseline})\]. Rate of rise was calculated as \((R_{\text{peak}} - R_{\text{baseline}}) / (time at R_{\text{peak}} \text{ time at R_{\text{baseline}}})\). Significance was determined using the Student’s two-sided \(t\) test.

### Results

**Bortezomib treatment induces the expression of gene products associated with the endoplasmic reticulum secretory pathway.** To investigate the mechanism of bortezomib-mediated cell death, oligonucleotide microarray analysis was used to examine the differential gene expression profile of the 8226 myeloma cell line following exposure to bortezomib. These studies were designed to examine the acute response of myeloma cells to bortezomib. Therefore, myeloma cells were treated with a short-term (4-hour) exposure using a dose corresponding to the approximate IC_{70} for a 24-hour exposure as determined by MTT and Annexin V-FITC. No phenotypic indications of cell death are visible at this time point. Using untreated cells as baseline, 25 gene products were identified as significantly expressed (fluorescence signal >200) and increased by >2-fold in cells maintained in suspension, whereas only 8 were decreased at the level of RNA expression. The most consistent and dramatic increase occurred in gene products associated with the ER secretory pathway (Table 1). Specifically, two isoforms of the chaperone 70 kDa heat shock protein (hsp70), 1A and 1B, were induced by 2.6- and 2.9-fold, respectively. The hsp70-interacting protein, BCL2-associated athanogene 3, was similarly induced 2.0-fold, and the luminal ER Ca^{2+}-regulating protein, calreticulin, was induced by 2.0-fold. Protein expression of these gene products was confirmed by Western blot analysis (data not shown).

**Caspase 12 is activated by bortezomib treatment.** One of the mechanisms proposed to contribute to ER stress–mediated death is the activation of the ER-resident caspase 12 (22). Caspase 12 is localized on the cytoplasmic side of the ER. It has been shown to be proteolytically activated by agents that induce ER stress, including tunicamycin, thapsigargin, brefeldin A (23), and m-calpain (24). Manganese(II) (25), \(\beta\)-amyloid protein, and disruption of Ca^{2+} homeostasis (24) are also reported to activate caspase 12. Examination of caspase 12 activation in 8226 myeloma cells following drug treatment with a variety of cytotoxic agents showed that bortezomib activates caspase 12 in myeloma cells, whereas other cytotoxic agents, including doxorubicin, melphalan, and CD95 ligation, did not (Fig. 1). This is not due to absence of drug-induced cytotoxicity, as analysis of apoptosis by Annexin V-FITC and flow cytometry showed comparable cell death in all drug-treated samples (data not shown). Additionally, examination of additional caspase cleavage products identified the predicted cleavage of caspase 9 in all experimental conditions, caspase 8 by CD95 cross-linking, and caspases 7 and 3 by all cytotoxic agents. Interestingly, caspase 8 was also cleaved by bortezomib treatment, demonstrating the involvement of apoptotic mediators typically associated with the extrinsic pathway.

**Caspase 8 modulates the activation of caspase 12.** Caspase activation has been shown to occur in a relatively linear sequence, and specific tetrapeptide inhibitors can be used to determine hierarchical ordering. To identify the apical caspase in bortezomib-mediated cytotoxicity, 8226 myeloma cells were treated with 50 nmol/L bortezomib in the presence and absence of the caspase 3 inhibitor DEVD, the caspase 8 inhibitor IETD, or the pan caspase inhibitor Z-VAD. All reagents were FMK conjugated for cell permeability, and Z-FA-FMK was used as a negative control. As shown in Fig. 2, 50 nmol/L bortezomib-induced activation of caspases 12, 3, 8, and 9, and was not inhibited by FA-FMK. The caspase 3 inhibitor DEVD partially prevented the cleavage of caspase 3, but had no effect on caspase 12, 8, or 9, and only slightly inhibited cell death. In contrast, the caspase 8 inhibitor IETD reduced the cleavage of

### Table 1. Oligonucleotide analysis of gene expression in myeloma cells following 4 hours of treatment with 50 nmol/L bortezomib

<table>
<thead>
<tr>
<th>Fold change</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>butyrate response factor 2 (epidermal growth factor-response factor 2)</td>
</tr>
<tr>
<td>0.4</td>
<td>homologue of murine interferon-inducible protein p78, MX1</td>
</tr>
<tr>
<td>0.4</td>
<td>multiple membrane spanning receptor TCR8</td>
</tr>
<tr>
<td>0.4</td>
<td>IFN-stimulated protein, 15 kDa (ISG15)</td>
</tr>
<tr>
<td>0.4</td>
<td>IFN-stimulated transcription factor 3, (\gamma)</td>
</tr>
<tr>
<td>0.5</td>
<td>IFN regulatory factor 7 (IRF7), transcript variant c</td>
</tr>
<tr>
<td>0.5</td>
<td>chromosome 19, cosmids R23184</td>
</tr>
<tr>
<td>0.5</td>
<td>nuclear receptor subfamily 4, group A, member 2</td>
</tr>
<tr>
<td>2.0</td>
<td>BCL2-associated athanogene 3 (BAG3)</td>
</tr>
<tr>
<td>2.0</td>
<td>calreticulin</td>
</tr>
<tr>
<td>2.0</td>
<td>KIAA0648 protein</td>
</tr>
<tr>
<td>2.0</td>
<td>prominin (mouse)-like 1 (PROML1)</td>
</tr>
<tr>
<td>2.1</td>
<td>basic-leucine zipper nuclear factor (JEM-1)</td>
</tr>
<tr>
<td>2.1</td>
<td>IFN-related developmental regulator 1</td>
</tr>
<tr>
<td>2.1</td>
<td>chondroitin sulfate proteoglycan 2 (versican)</td>
</tr>
<tr>
<td>2.3</td>
<td>cDNA DKEFP43HM054</td>
</tr>
<tr>
<td>2.3</td>
<td>cDNA FLJ11868 fis, clone HEMBA1006993</td>
</tr>
<tr>
<td>2.4</td>
<td>chromosome 16 open reading frame 7</td>
</tr>
<tr>
<td>2.4</td>
<td>shc pseudogene, p66 isof orm</td>
</tr>
<tr>
<td>2.5</td>
<td>FLJ100032 protein, partial cds</td>
</tr>
<tr>
<td>2.6</td>
<td>heat shock 70 kDa protein 1A (HSPA1A)</td>
</tr>
<tr>
<td>2.7</td>
<td>hypothetical protein FLJ20063</td>
</tr>
<tr>
<td>2.8</td>
<td>TATA box binding protein (TBP)-associated factor, RNA polymerase II (TAF2)</td>
</tr>
<tr>
<td>2.9</td>
<td>VAMP-associated protein of 33 kDa mRNA</td>
</tr>
<tr>
<td>2.9</td>
<td>heat shock 70 kDa protein 1B (HSPA1B)</td>
</tr>
<tr>
<td>3.0</td>
<td>ribosomal protein L39</td>
</tr>
<tr>
<td>3.1</td>
<td>ATP-binding cassette, subfamily F (GCN20), member 2 (ABC2F)</td>
</tr>
<tr>
<td>3.2</td>
<td>hypothetical protein FLJ21032 (FLJ21032)</td>
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<tr>
<td>3.2</td>
<td>cDNA DKEFP686H072</td>
</tr>
<tr>
<td>3.7</td>
<td>cDNA FLJ13829 fis, clone THYBO1000625</td>
</tr>
<tr>
<td>3.7</td>
<td>EST pseudogene similar to UBL1 [ubiquitin-like 1 (sentrin)]</td>
</tr>
<tr>
<td>4.8</td>
<td>Sec23-interacting protein p125</td>
</tr>
<tr>
<td>5.9</td>
<td>EST for novel 7 transmembrane receptors</td>
</tr>
</tbody>
</table>

NOTE: Gene products known to be associated with the ER secretory pathway are highlighted.
caspases 12, 3, and to a lesser extent, caspase 9, and reduced Annexin V-FITC staining by nearly 50%, suggesting that the activation of caspase 8 is at least one of the primary initiating caspases in bortezomib-mediated cell death.

Inhibition of mitochondrial Ca\(^{2+}\) uptake prevents bortezomib-induced cytotoxicity. Intracellular Ca\(^{2+}\) is a second messenger that has been associated with caspase 12 activation and regulates a large number of processes, including apoptosis (see review in ref. 10). To determine if disregulation of intracellular Ca\(^{2+}\) contributes to bortezomib-mediated cytotoxicity, drug activity was examined in the presence of a series of Ca\(^{2+}\)-interacting agents. These studies were based on the hypothesis that if disregulation of intracellular Ca\(^{2+}\) is required for bortezomib-mediated cell death, then inhibition of intracellular Ca\(^{2+}\) homeostasis using a panel of pharmacologic inhibitors would alter the response of myeloma cells to bortezomib-induced cell death. Calcium release from the ER is regulated by two primary receptors, the IP3R and the ryanodine receptor. The ryanodine receptor can be inhibited by dantrolene, which is used clinically as a muscle relaxant to block Ca\(^{2+}\) release from the sarcoendoplasmic reticulum. 2-APB was originally described as an IP3R inhibitor (26), although more recent data have shown that 2-APB is also a potent blocker of capacitative Ca\(^{2+}\) entry channels independent of its inhibition of the IP3R (27, 28).

Free cytosolic Ca\(^{2+}\) can be lowered by incubation with the Ca\(^{2+}\) chelating agent, BAPTA-AM. Finally, mitochondrial uptake of Ca\(^{2+}\) via the mitochondrial uniporter can be inhibited by ruthenium red (29) or its dinuclear analog, Ru360 (30). Preliminary experiments showed that neither ruthenium red nor Ru360 induced significant cell death at concentrations up to 100 \(\mu\)mol/L (data not shown). All other inhibitors were used at doses that resulted in less than 15% cell death in control experiments. 8226 myeloma cells were simultaneously exposed to bortezomib in the presence or absence of minimally toxic doses of each Ca\(^{2+}\)-interacting agent, and cytotoxicity was analyzed by MTT dye reduction. As shown in Fig. 3A, cotreatment of 8226 cells with the mitochondrial Ca\(^{2+}\) uptake inhibitor ruthenium red (0.1 \(\mu\)mol/L) or Ru360 (1.0 \(\mu\)mol/L) almost entirely abrogated bortezomib-induced cytotoxicity. Identical results were obtained for additional cell lines, including the myeloma cells H929, U266, and MM.1S, diffuse large cell lymphoma cells DB and Raji, and the mantle cell lymphoma cell line Granta-1 (data not shown). No significant inhibition of bortezomib-mediated cytotoxicity was seen in cells cotreated with agents that inhibit Ca\(^{2+}\) efflux from the ER or with the Ca\(^{2+}\) chelator BAPTA-AM (Fig. 3B). Increasing concentrations of 2-APB up to 100 \(\mu\)mol/L did not produce any additional activity, nor did dantrolene at

Figure 1. Caspase activation in 8226 myeloma cells by various cytotoxic stimuli. 8226 myeloma cells were treated with the indicated agent for 16 hours, harvested, and 30 \(\mu\)g of total protein were analyzed by Western blot for activated caspase cleavage products. Concurrent analysis of cell death by Annexin V-FITC staining showed 50% to 70% apoptosis in all samples (data not shown). Representative of three independent experiments.

Figure 2. Inhibition of bortezomib-mediated caspase cleavage by tetrapeptide inhibitors. 8226 myeloma cells were incubated with 50 nmol/L of the indicated tetrapeptide inhibitor for 30 minutes before 16-hour treatment with 50 nmol/L bortezomib. Cells were harvested and 30 \(\mu\)g of total protein were analyzed by Western blot for caspase cleavage products. Viability was determined by Annexin V-FITC staining and flow cytometric analysis of cell aliquot before lysis. \(\beta\)-Actin is used to show equal protein loading. Representative of three independent experiments.
and it is possible that the concentration used for these experiments significantly toxic to myeloma cells, with 30% apoptosis at 5 mol/L. Preliminary control experiments showed that BAPTA-AM is required for Ca²⁺ disregulation, caspase activity was verified by Annexin V-FITC staining and flow cytometry analysis. Identical results were obtained (data not shown). Additionally, 4-day MTT analysis shows that myeloma cells treated with bortezomib in the presence of 2-APB, dantrolene, BAPTA-AM, or ruthenium red and Ru360 for 24 hours, and cell viability analyzed by MTT dye reduction assay. Data are expressed as (mean absorbance treated / mean absorbance control) × 100, where control is ruthenium red, Ru360, or untreated. B. 24-hour MTT dye reduction analysis of 8226 myeloma cells treated with bortezomib in the presence or absence of 5 μmol/L 2-APB, 10 μmol/L dantrolene, or 5 μmol/L BAPTA-AM. Preliminary experiments showed less than 10% cytotoxicity with 5 μmol/L 2-APB and 10 μmol/L dantrolene. BAPTA-AM induces 30% cytotoxicity at 5 μmol/L. Data are expressed as (mean absorbance treated / mean absorbance control) × 100, where control is 2-APB, dantrolene, BAPTA-AM, or untreated. The effects of ruthenium red (RR) on thapsigargin cytotoxicity were determined exactly as in A. Columns, mean of at least three independent experiments; *, P < 0.05.

Figure 3. Effects of Ca²⁺-modulating agents on bortezomib cytotoxicity. A, 8226 myeloma cells were exposed to the indicated concentration of bortezomib in the presence or absence of 0.1 μmol/L ruthenium red or 1.0 μmol/L Ru360 for 24 hours, and cell viability analyzed by MTT dye reduction assay. Data are expressed as (mean absorbance treated / mean absorbance control) × 100, where control is ruthenium red, Ru360, or untreated. B. 24-hour MTT dye reduction analysis of 8226 myeloma cells treated with bortezomib in the presence or absence of 5 μmol/L 2-APB, 10 μmol/L dantrolene, or 5 μmol/L BAPTA-AM. Preliminary experiments showed less than 10% cytotoxicity with 5 μmol/L 2-APB and 10 μmol/L dantrolene. BAPTA-AM induces 30% cytotoxicity at 5 μmol/L. Data are expressed as (mean absorbance treated / mean absorbance control) × 100, where control is 2-APB, dantrolene, BAPTA-AM, or untreated. The effects of ruthenium red (RR) on thapsigargin cytotoxicity were determined exactly as in A. Columns, mean of at least three independent experiments; *, P < 0.05.

To determine if the mitochondrial uniporter is involved in cell death induced by standard chemotherapeutic drugs, myeloma cells were treated with a variety of cytotoxic agents representing diverse mechanisms of activity in the presence and absence of 0.1 μmol/L ruthenium red. Cotreatment with ruthenium red has no effect on the cytotoxic activity of the topoisomerase II inhibitors doxorubicin and mitoxantrone, alkylating agents melphalan and cisplatinum, or the mitochondrial targeting agents imexon (31) and dexamethasone (data not shown). Additionally, the cytotoxic effect of thapsigargin, which is an irreversible inhibitor of the smooth ER Ca²⁺-ATPase and induces Ca²⁺ store depletion, is not inhibited by ruthenium red (Fig. 3B). These data show the dramatic specificity of the inhibitory effects of ruthenium-containing agents on bortezomib-induced death and suggest that the critical determinant of bortezomib cytotoxicity is the mitochondrial-dependent disregulation of Ca²⁺.

Mitochondrial Ca²⁺ disregulation occurs upstream of caspase activation. To determine if mitochondrial Ca²⁺ disregulation was required to initiate caspase activation or, conversely, if caspase activity was required for Ca²⁺ disregulation, caspase activity was examined in the presence of the Ca²⁺-modulating agents. 8226 myeloma cells were cotreated with 50 nmol/L PS-341 in the presence or absence of 2-APB, BAPTA-AM, or ruthenium red and examined for caspase activation. Similar to Fig. 1, bortezomib alone induces significant caspase activation (Fig. 4). Inhibition of the mitochondrial Ca²⁺ uniporter with 0.1 μmol/L ruthenium red entirely prevented activation of caspases 12, 8, and 3 and cleavage of bid. Cotreatment with 1.0 μmol/L Ru360 gave identical results (data not shown). In contrast, 2-APB and BAPTA-AM had no effect. These data further support the hypothesis that mitochondrial Ca²⁺ is the critical determinant of bortezomib-mediated cytotoxicity. Furthermore, mitochondrial Ca²⁺ disregulation occurs before caspase activation, including caspase 8, which is typically considered upstream of mitochondrial permeability transition.

Ca²⁺ homeostasis is disregulated following bortezomib treatment. To identify the effectors of Ca²⁺ homeostasis that may be contributing to bortezomib-mediated cytotoxicity, imaging of cytosolic Ca²⁺ was done using the ratiometric dye Fura-AM. Initial concentrations up to 50 μmol/L (data not shown). However, preliminary control experiments showed that BAPTA-AM is significantly toxic to myeloma cells, with 30% apoptosis at 5 μmol/L, and it is possible that the concentration used for these experiments may not have been sufficient to entirely deplete cytosolic Ca²⁺. To rule out the possibility that ruthenium red and Ru360 were affecting the activity of mitochondrial succinate dehydrogenase, which is required for MTT dye reduction, apoptosis was verified by Annexin V-FITC staining and flow cytometry analysis. Identical results were obtained (data not shown). Additionally, 4-day MTT analysis shows that myeloma cells treated with bortezomib in the presence of ruthenium-containing agents continue to proliferate, suggesting that inhibition of the mitochondrial uniporter does not only delay apoptosis but prevents cell death.

To determine if the mitochondrial uniporter is involved in cell death induced by standard chemotherapeutic drugs, myeloma cells were treated with a variety of cytotoxic agents representing diverse mechanisms of activity in the presence and absence of 0.1 μmol/L ruthenium red. Cotreatment with ruthenium red has no effect on the cytotoxic activity of the topoisomerase II inhibitors doxorubicin and mitoxantrone, alkylating agents melphalan and cisplatinum, or the mitochondrial targeting agents imexon (31) and dexamethasone (data not shown). Additionally, the cytotoxic effect of thapsigargin, which is an irreversible inhibitor of the smooth ER Ca²⁺-ATPase and induces Ca²⁺ store depletion, is not inhibited by ruthenium red (Fig. 3B). These data show the dramatic specificity
studies examining the acute affects of bortezomib exposure showed that bortezomib induces a rapid and transient increase in cytosolic Ca\(^{2+}\), which quickly recovers to basal levels (Fig. 5A). Cytosolic Ca\(^{2+}\) peaked within 5 to 8 minutes of treatment, with complete recovery to baseline occurring by 15 minutes in all cells analyzed. This transient increase in [Ca\(^{2+}\)]\(_i\) following bortezomib treatment may be due to release of Ca\(^{2+}\) from intracellular stores and/or influx from the extracellular space. Subsequent addition of cyclopiazic acid, to induce store unloading, elicited another large transient increase in [Ca\(^{2+}\)]\(_i\), indicating that significant levels of Ca\(^{2+}\) remained within the stores following bortezomib exposure (Fig. 5A).

To investigate the potential mechanism for the bortezomib effects on [Ca\(^{2+}\)]\(_i\), 8226 cells were incubated with 500 nmol/L PS-341 for 2 hours before analysis of [Ca\(^{2+}\)]\(_i\). Cytosolic Ca\(^{2+}\) peaked within 5 to 8 minutes of treatment, with complete recovery to baseline occurring by 15 minutes in all cells analyzed. This transient increase in [Ca\(^{2+}\)]\(_i\), following bortezomib treatment may be due to release of Ca\(^{2+}\) from intracellular stores and/or influx from the extracellular space. Subsequent addition of cyclopiazic acid, to induce store unloading, elicited another large transient increase in [Ca\(^{2+}\)]\(_i\), indicating that significant levels of Ca\(^{2+}\) remained within the stores following bortezomib exposure (Fig. 5A).

To investigate the potential mechanism for the bortezomib effects on [Ca\(^{2+}\)]\(_i\), 8226 cells were incubated with 500 nmol/L PS-341 for 2 hours before analysis of [Ca\(^{2+}\)]\(_i\). At this time following bortezomib treatment, mitochondrial membrane potential remains intact, as determined by control experiments using the mitochondrial dye CMXRos as well as by Western blotting for cytochrome c release (data not shown). A standard cyclopiazic acid response protocol was used to determine if store Ca\(^{2+}\) was altered following bortezomib treatment. Plasma membrane influx and efflux of Ca\(^{2+}\) must be eliminated to effectively compare stored Ca\(^{2+}\) in control and treated cells. To do this, cells were perfused in Ca\(^{2+}\)- and Na\(^{+}\)-free HBSS with 1 μmol/L vanadate to block Ca\(^{2+}\) influx and the activities of the Na\(^{+}\)-Ca\(^{2+}\) exchanger and plasma membrane Ca\(^{2+}\)-ATPase, respectively. Treatment of 8226 cells with bortezomib for 2 hours had no significant effect on the level of stored Ca\(^{2+}\) (Figs. 5B and 6A), again indicating that if bortezomib acutely altered store Ca\(^{2+}\), this was a transient effect.
with Ru360 in the presence of Ca2+ efflux inhibitors significantly regulating the efflux from or loading of Ca2+ into the ER (Fig. 6). The measured [Ca2+]i following CCI in myeloma cells exposed to 500 nmol/L bortezomib attained mean levels of 0.73 ± 0.09 μmol/L (SD 0.089) compared with 0.56 ± 0.04 μmol/L (SD 0.043) in control cells (P = 0.05). Concurrent treatment with Ru360 abrogated the [Ca2+]i to 0.49 ± 0.04 μmol/L (SD 0.032) in control cells, suggesting that inhibition of the mitochondrial uniporter normalized the effect of bortezomib on the overall Ca2+ fluxes. These data are in accordance with previous studies demonstrating that mitochondria are an active participant in the maintenance of cellular Ca2+ homeostasis and in the activation and extent of CCI (10).

**Discussion**

The boronic acid dipeptide bortezomib is a small molecule inhibitor of the 20S proteasome that has shown antitumor activity in a number of cell types, and is particularly effective in the treatment of patients with multiple myeloma. The findings presented here show a unique apoptotic pathway initiated by bortezomib that uses Ca2+ to activate both intrinsic and extrinsic mediators.

The ubiquitin-proteasome system of protein degradation plays an essential role in quality control of newly synthesized proteins. The data presented here show that bortezomib induces the expression of proteins associated with the ER secretory pathway, indicative of potential ER stress-initiated pathway to apoptosis. While these studies were in progress, two additional studies reported gene expression profiling in cells treated with proteasome inhibitors. Fleming et al. (32) examined the profile of *Saccharomyces cerevisiae* treated with bortezomib or β-lactone, whereas Mitsiades et al. (33) reported the profile of the human multiple myeloma cell line MM.1S following exposure to 100 nmol/L bortezomib. These data are highly compatible; as in all three studies, the most significant and dramatic induction is that of proteins associated with the ER secretory pathway, suggesting that the mechanism of bortezomib cytotoxicity involves ER stress.

As with many cellular stress responses, if the level of stress exceeds the adaptive capacity of the cell, an apoptotic pathway is initiated. ER stress–mediated apoptosis has been associated with the activation of caspase 12 (23, 34, 35), although the existence of caspase 12 in humans is somewhat controversial. Caspase 12 was originally identified in a murine system, and Fischer et. al. (36) reported a human caspase 12 sequence that is predicted to encode multiple splice variants and stop codons. Antibodies directed against a conserved region of the mouse protein identify a human cellular protein that is of similar relative molecular mass to 1.6 mmol/L (C2). Intracellular Ca2+ concentration is calculated based on a standard curve as previously described. C. rate of rise, CCI response. Data are calculated as \[(R_{\text{peak}} - R_{\text{baseline}}) / (\text{time at } R_{\text{peak}} - \text{time at } R_{\text{baseline}})\]. Ru360 also significantly and substantially decreased the rate and magnitude of CCI in both untreated controls and bortezomib-treated cells (Fig. 6). Moreover, the peak [Ca2+]i, attained in cells treated with bortezomib in the presence of Ru360 was significantly less than that seen with bortezomib alone (Fig. 6B). The measured [Ca2+]i, following CCI in myeloma cells exposed to 500 nmol/L bortezomib attained mean levels of 0.73 ± 0.09 μmol/L (SD 0.089) compared with 0.56 ± 0.04 μmol/L (SD 0.043) in control cells (P = 0.05). Concurrent treatment with Ru360 abrogated the [Ca2+]i, to 0.49 ± 0.04 μmol/L (SD 0.032) in control cells, suggesting that inhibition of the mitochondrial uniporter normalized the effect of bortezomib on the overall Ca2+ fluxes. These data are in accordance with previous studies demonstrating that mitochondria are an active participant in the maintenance of cellular Ca2+ homeostasis and in the activation and extent of CCI (10).

**Figure 6.** Analysis of the regulation of [Ca2+]i following 2 hours of bortezomib treatment. Myeloma cells were treated with 500 nmol/L bortezomib in the presence or absence of 1.0 μmol/L Ru360 for 2 hours before loading with Fura-2 dye and fluorescence analysis (see Materials and Methods). A, change in Ca2+ recorded after addition of cyclopiazonic acid. B, peak [Ca2+]i attained following elevation of extracellular Ca2+ to 1.6 mmol/L (CCI). Intracellular Ca2+ concentration is calculated based on a standard curve as previously described. C, rate of rise, CCI response. Data are calculated as \[(R_{\text{peak}} - R_{\text{baseline}}) / (\text{time at } R_{\text{peak}} - \text{time at } R_{\text{baseline}})\].
upstream of caspase activation and bid cleavage. Inhibition of the mitochondrial uniporter with ruthenium-containing compounds entirely abrogated bortezomib-induced caspase activation. In contrast, Darios et al. (38) showed that mitochondrial Ca²⁺ uptake is a consequence of these events in a model system of ceramide-mediated apoptosis in neurally differentiated PC12 cells. Additionally, in this study, they were unable to inhibit ceramide-induced cell death with ruthenium-containing compounds, suggesting that the mechanism of Ca²⁺ disruption incurred by ceramide is distinct from the cellular response to proteasome inhibition.

The regulation of Ca²⁺ signaling and Ca²⁺-dependent proteins has long been associated with apoptosis, but only recently have their roles been defined and the regulatory mechanisms begun to be identified. The regulation of apoptosis by Ca²⁺ signaling was first shown with the identification of a Ca²⁺/Mg²⁺-dependent endonuclease responsible for the DNA fragmentation, which is often considered the hallmark of apoptosis (39). Increased cytosolic Ca²⁺ concentration has been found to subsequently activate Ca²⁺-dependent enzymes that modulate cell death, including p-calpain and calcineurin (40). Furthermore, Ca²⁺ influx into mitochondrial, if substantial, can activate cytochrome c release, which subsequently activates caspase activity (15). The lumen of the ER is the primary storage location for Ca²⁺, where it is either free or bound to luminal proteins such as calreticulin, an unfolded protein-responsive chaperone that we found to be induced by 2-fold following bortezomib treatment. In this respect, Arnaudeau et al. (41) showed that increased expression of calreticulin resulted in an increased rate of agonist-induced Ca²⁺ release and reduced mitochondrial Ca²⁺ retention. Although a functional role for calreticulin in bortezomib-mediated cell death has not yet been defined, the observed elevation of mRNA and protein levels suggests that calreticulin expression may be induced as a cellular response to ER stress, and therefore alterations in ER Ca²⁺ storage or release may play a role in the observed disruption of Ca²⁺ homeostasis. However, the data shown in Figs. 5 and 6 clearly show that releasable store Ca²⁺ levels are not substantially different in cells treated with bortezomib. The primary difference in Ca²⁺ homeostasis observed in bortezomib-treated cells was a significant elevation in cytoplasmic acid–activated CCI (Figs. 5 and 6), indicating an increased potential for Ca²⁺ influx following store release. Interestingly, inhibition of mitochondrial Ca²⁺ uptake by ruthenium red significantly reduced ER Ca²⁺ loading and the bortezomib enhancement of store-activated influx. The mechanism by which depletion of ER Ca²⁺ activates plasma membrane channels is unclear; however, one model suggests that mitochondria play an important role in regulating both the loading state of Ca²⁺ stores (42) and the coupling of store Ca²⁺ released for channel activation (43). We propose a model where bortezomib evokes a transient release of Ca²⁺ stores leading to mitochondrial Ca²⁺ influx. Mitochondrial Ca²⁺ sensors associated with the uniporter initiate CCI, which is enhanced in bortezomib-treated cells (Fig. 6), leading to caspase activation. Ruthenium compounds would then be protective by blocking mitochondrial loading and CCI activation and the Ca²⁺-dependent signal transduction pathways that initiate cell death (44). The identity of the signal pathways involved in this regulation remains under intense investigation. Further definition of the mediators in this pathway will promote the design of strategies to enhance drug activity, reduce toxicity, and overcome drug resistance.

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Mitochondrial-Mediated Disregulation of Ca$^{2+}$ Is a Critical Determinant of Velcade (PS-341/Bortezomib) Cytotoxicity in Myeloma Cell Lines

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