Mitochondrial-Mediated Disregulation of Ca\(^{2+}\) 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\(^{2+}\) as a mechanism of caspase activation. Cotreatment with a panel of Ca\(^{2+}\)-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\(^{2+}\)-modulating agents, including 2-amino-ethoxydiphenylborate, 1,2-bis (o-aminophenoxy) ethane-tetraacetic acid (acetoxy-methyl) ester, and dantrolene, did not alter bortezomib cytotoxicity, and regulating 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\(^{2+}\)-modulating agents, including 2-amino-ethoxydiphenylborate, 1,2-bis (o-aminophenoxy) ethane-tetraacetic acid (acetoxy-methyl) ester, and dantrolene, did not alter bortezomib cytotoxicity, and regulating agents identified the mitochondrial uniporter as a critical regulatory factor in bortezomib cytotoxicity.

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. 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\(_{L}\) 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\(^{2+}\) homeostasis.

Disregulation of intracellular Ca\(^{2+}\) was among the first hallmarks 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\(^{2+}\) associated with apoptosis: first, a transient spike occurring immediately following the cellular insult; second, sustained Ca\(^{2+}\) influx that was considered to be the lethal event. Subsequent studies have shown that even minor disruptions in either total Ca\(^{2+}\) or subcellular distribution can modulate the apoptotic
response to a large number of stimuli. In most cell types, the ER is the primary intracellular store of Ca\(^{2+}\), where it participates in the folding, modification, and sorting of newly synthesized proteins. Homeostasis between Ca\(^{2+}\) stores and cytosolic Ca\(^{2+}\) is maintained by ER-resident channels and transporters. The primary mechanism for ER Ca\(^{2+}\) influx is the smooth ER Ca\(^{2+-}\)ATPase, which mediates store filling. Two primary Ca\(^{2+}\)-sensitive channels mediate the release of Ca\(^{2+}\) from the ER, the inositol triphosphate receptor (IP3R) and the caffeine sensitive ryanodine receptor. Recent studies suggest that IP3R-deactivated depletion of ER stores is also involved in the regulation of Ca\(^{2+}\) influx from the extracellular environment, a phenomenon known as capacitative Ca\(^{2+}\) influx (CCI, ref. 11). Vazquez et al. showed that a genetic variant of DT40 B lymphocytes deficient in IP3R expression was unable to transport Ca\(^{2+}\) 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 Ca\(^{2+}\), supporting a role for CCI in apoptosis.

In addition to their function in metabolism and apoptosis, mitochondria are known to participate in Ca\(^{2+}\) homeostasis. Excess cytosolic Ca\(^{2+}\) is taken up into the mitochondria through a low-affinity inner membrane Ca\(^{2+}\) uniporter and released back to the cytosol by three independent mechanisms: reversal of the uniporter, Na\(^+\)/H\(^-\)-dependent Ca\(^{2+}\) exchange, or the mitochondrial permeability transition pore opening. Additional studies have shown IP3R-mediated translocation of Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 disregulation of Ca\(^{2+}\) homeostasis leading to cell death. We further show that inhibition of mitochondrial Ca\(^{2+}\) uptake by ruthenium compounds completely abrogates the cytotoxic activity of bortezomib, whereas a series of Ca\(^{2+}\)-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 Ca\(^{2+}\) inhibitor plus cytotoxic agent, the inhibitor was added to the total cell population before plating. Cytotoxic activity of the Ca\(^{2+}\)-modulating agents alone was determined in preliminary control experiments, and all subsequent assays were done at minimal toxic doses as follows: 2-amino-ethoxydiphosphorylborate (2-APB), 5 mmol/L; 1,2-bis-(o-aminophenoxy)ethane-tetraacetic acid (acetoxymethyl) ester (BAPTA-AM), 10 mmol/L; dantrolene, 10 mmol/L; Ru360, 1.0 mmol/L; and ruthenium red, 0.1 mmol/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 MgCl\(_2\), 25 mmol/L NaF, 1 mmol/L EGTA, 1% Triton X-100, 10% glycerol with 2 mmol/L NaVO\(_4\), 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, and 2 mmol/L phenylmethylsulfonyl 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 polyvinylidine difluoride membrane (BioRad, Hercules, CA), probed with the specified antibody, and developed using Pierce Supersignal chemiluminescence substrate.

**[Ca\(^{2+}\)]\(_i\) measurements.** For [Ca\(^{2+}\)]\(_i\) measurements, cells were pelleted to #1 round glass coverslips coated with poly-n-lysine (Sigma) or fibronectin (Roche, Indianapolis, IN) with identical results on both substrates. Individual coverslips were incubated in a buffered saline (5 mmol/L KCl, 0.3 mmol/L KH\(_2\)PO\(_4\), 138 mmol/L NaCl, 0.2 mmol/L NaHCO\(_3\), 0.3 mmol/L Na\(_2\)HPO\(_4\), 20 mmol/L HEPES, 1.3 mmol/L CaCl\(_2\), 0.4 mmol/L MgSO\(_4\), and 5.6 mmol/L glucose, pH 7.3, at 37°C) containing 2.5 μmol/L Fura-PE3/AM (Te'dalabs, 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 bandpass filter centered at 510 nm and passed to a CCD camera.
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>
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<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 (IFN-inducible protein p78; MX1)</td>
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<tr>
<td>0.4</td>
<td>multiple membrane spanning receptor TRC8</td>
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<tr>
<td>0.4</td>
<td>IFN-stimulated protein, 15 kDa (ISG15)</td>
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<td>0.4</td>
<td>IFN-stimulated transcription factor 3, γ (48 kDa; ISGF3G)</td>
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<tr>
<td>0.5</td>
<td>IFN regulatory factor 7 (IRF7), transcript variant c</td>
</tr>
<tr>
<td>0.5</td>
<td>chromosome 19, cosmid R32184</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>
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<tr>
<td>2.0</td>
<td>calreticulin</td>
</tr>
<tr>
<td>2.0</td>
<td>KIAA0648 protein</td>
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<tr>
<td>2.0</td>
<td>promin (mouse)-like 1 (PROML1)</td>
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<td>basic-leucine zipper nuclear factor (JEM-1)</td>
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<tr>
<td>2.1</td>
<td>IFN-related developmental regulator 1</td>
</tr>
<tr>
<td>2.3</td>
<td>chondroitin sulfate proteoglycan 2 (versican)</td>
</tr>
<tr>
<td>2.3</td>
<td>cDNA DKFZp434M054</td>
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<td>cDNA FLJ11868 fis, clone HEMBA1006993</td>
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<td>2.4</td>
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<td>FLJ00032 protein, partial cds</td>
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<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>
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NOTE: Gene products known to be associated with the ER secretory pathway are highlighted.

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 IC70 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 Ca2+-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), β-amyloid protein, and disruption of Ca2+ 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...
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 sarcoplasmatic 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$^{2+}$ chelator 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 μmol/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 μmol/L) or Ru360 (1.0 μmol/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 μmol/L did not produce any additional activity, nor did dantrolene at...
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 Ca^{2+}. 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 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 deregulation of Ca^{2+}.

Mitochondrial Ca^{2+} disgregation occurs upstream of caspase activation. To determine if mitochondrial Ca^{2+} disgregation was required to initiate caspase activation or, conversely, if caspase activity was required for Ca^{2+} disgregation, caspase activity was examined in the presence of the Ca^{2+}-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 Ca^{2+} uniporter with 0.1 μmol/L ruthenium red entirely prevented activation of caspases 12, 8, and 3 and cleavage of bid. Cotreatment with 0.1 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 Ca^{2+} is the critical determinant of bortezomib-mediated cytotoxicity. Furthermore, mitochondrial Ca^{2+} disgregation occurs before caspase activation, including caspase 8, which is typically considered upstream of mitochondrial permeability transition.

Ca^{2+} homeostasis is disregulated following bortezomib treatment. To identify the effectors of Ca^{2+} homeostasis that may be contributing to bortezomib-mediated cytotoxicity, imaging of cytosolic Ca^{2+} was done using the ratiometric dye Fura-AM. Initial
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 cyclopiazoic 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}. 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 cyclopiazoic acid 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.

Depletion of Ca^{2+} stores with agents such as thapsigargin or cyclopiazoic acid activates store-operated channels, and thereby CCI. To examine the extent of CCI following treatment with cyclopiazoic acid, the media Ca^{2+} was increased to 1.3 mmol/L in the presence of Ca^{2+} efflux inhibitors and [Ca^{2+}]_{i} was analyzed. As shown in Fig. 5B (t = 11-14 min) and Fig. 6B and C, both the

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**Figure 4.** Effects of Ca^{2+}-modulating agents on caspase activation. 8226 myeloma cells were treated for 16 hours with 50 nmol/L bortezomib in the presence or absence of 5 μmol/L 2-APB, 5 μmol/L BAPTA-AM, or 0.1 μmol/L ruthenium red, harvested, and 30 μg 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. Representative of three independent experiments.

**Figure 5.** Effects of bortezomib on Ca^{2+} homeostasis. A, acute effects of bortezomib on cytosolic Ca^{2+}. 8226 myeloma cells were immobilized on coverslips and loaded with Fura-2 ratiometric dye before treatment with 50 or 500 nmol/L bortezomib. Following recovery from the bortezomib response, 5 μmol/L ionomycin was added to equilibrate Ca^{2+} across the plasma membrane and to obtain a measure of Rmax. Representative time course of response of a single cell treated with 500 nmol/L bortezomib. The experiment was repeated thrice, with a minimum of 5 cells analyzed in each experiment. Similar data were obtained with 50 nmol/L bortezomib. B, effects of 2-hour bortezomib treatment on Ca^{2+} homeostasis. In the absence of external Ca^{2+} and the presence of Ca^{2+} efflux inhibitors, 30 μmol/L cyclopiazoic acid was added to assess the level of stored Ca^{2+}. As shown in B, the extracellular media was exchanged for Ca^{2+}- and Na^{+}-free HBSS supplemented with 1 μmol/L vanadate to block Ca^{2+} influx and efflux pathways. After 2 minutes following the reduction in cytosolic Ca^{2+}, 30 μmol/L cyclopiazoic acid (CPA) was added to release Ca^{2+} from internal stores. Following recovery from the cyclopiazoic acid–induced increase in [Ca^{2+}], an equal volume of HBSS containing 2.6 mmol/L Ca^{2+} was added to the chamber, resulting in a physiologic extracellular Ca^{2+} concentration of 1.3 mmol/L, and the CCI was recorded. At the conclusion of the experiment, 5 μmol/L ionomycin was added to determine the Rmax. Points, mean of at least 5 cells analyzed in three independent experiments; bars, SE. No differences were seen in cells that adhered to fibronectin compared with those that adhered to poly-L-lysine. Control: dashed line; Bortezomib: solid line.
magnitude and rate of CCI increased in bortezomib-treated cells compared with controls (Fig. 6C), suggesting that bortezomib alters either the number of store-operated Ca^{2+} channels or the length of time such channels remain open following store depletion.

The effect of inhibiting the mitochondrial Ca^{2+} uniporter on the Ca^{2+} responses to cyclopiazic acid and the subsequent CCI were evaluated by incubating cells with ruthenium-containing compounds before analysis of [Ca^{2+}]i responses. Incubation of cells with Ru360 in the presence of Ca^{2+} efflux inhibitors significantly blunted the cyclopiazic acid response in both control and bortezomib-treated cells, suggesting a role for mitochondria in regulating the efflux from or loading of Ca^{2+} into the ER (Fig. 6).

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 [Ca^{2+}]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 [Ca^{2+}]i following CCI in myeloma cells exposed to 500 nmol/L bortezomib attained mean levels of 0.73 µmol/L (SD 0.089) compared with 0.56 µmol/L (SD 0.043) in control cells (P = 0.05). Concurrent treatment with Ru360 abrogated the [Ca^{2+}]i to 0.49 µmol/L (SD 0.053) with bortezomib exposure or 0.44 µmol/L (SD 0.032) in control cells, suggesting that inhibition of the mitochondrial uniporter normalized the effect of bortezomib on the overall Ca^{2+} fluxes. These data are in accordance with previous studies demonstrating that mitochondria are an active participant in the maintenance of cellular Ca^{2+} 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 Ca^{2+} 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, indicating a 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 caspase 12 antibody may cross-react with another as yet unidentified caspase, however, the activation of this caspase is clearly restricted to ER stress–initiated apoptosis. Several mechanisms of caspase 12 activation have been identified, and would seem to be cell type specific (37). The present study shows that bortezomib-mediated activation of caspase 12 may be modulated by caspase 8, but is dependent on mitochondrial Ca^{2+} disregulation. Our findings also show mitochondrial Ca^{2+} influx as an event that of proteins associated with the ER secretory pathway, resulting in ER stress.

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upstream of caspase activation and bid cleavage. Inhibition of the mitochondrial unipporter 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-inhibited 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 β-calpain and calcineurin (40). Furthermore, Ca²⁺ influx into mitochondria, 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 deregulation 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 cyclopiazonic 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 unipporter 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.

References
5. Ling YH, Liebes L, Jiang JD, et al. 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 β-calpain and calcineurin (40). Furthermore, Ca²⁺ influx into mitochondria, 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 deregulation 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 cyclopiazonic 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 unipporter 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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14. Jayaraman T, Marks AR. T cells deficient in intoxin 1,4,5-trisphosphate receptor are resistant to apoptosis. Mol Cell Biol 1997;17:3005–12.
24. Osbahrin H, Chock PB, Stadtman ER. Manganese(I) induces apoptotic cell death in NIH3T3 cells via a


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